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Viewpoint

New approaches build upon historical studies in dermal toxicology

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These are my personal reflections on the history of approaches to understanding dermal toxicology brought together for the Paton Prize Award. This is not a comprehensive account of all publications from in vivo studies in humans to development of in vitro and in silico approaches but highlights important progress. I will consider what is needed now to influence approaches to understanding dermal exposure with the current development and use of NAMs (new approach methodologies).

Key words: skin; future; prediction.

Historical development

Early observations involving skin included the effects of occupational exposure to chemicals and topical agents applied for therapeutic purposes. The classical observation of Sir Percivall Pott in 1775 described the occupational origin of scrotal cancer as "soot warts" in the scrotum of young boys who swept the soot from fireplace chimneys in England. This was later shown to be associated with dermal absorption of polyaromatic hydrocarbons.[1]

For centuries there has been interest in applying chemicals to skin with potential local and systemic effects leading to therapeutic or toxic outcomes. The 1940s to 70s were formative years in skin delivery understanding[2,3] Early investigators noticed toxic effects associated with skin application of chemicals and realized that there was absorption through the skin. For example Lindsey in Australia in 1968 observed fatal poisoning with topical salicylate[4] and Johnston[5] self-applied nicotine. To quote him: '*On one occasion I painted an area 3″ by 2″ on the flexor aspect of my forearm with nicotine 5%. In about 7 min I felt wretched, nauseated, headachy, faint and deduced that there had been dermal absorption*. Draize and coworkers in the 1940s investigated mechanisms of local skin irritation and toxicity from chemicals[6] and dermal metabolism.[7] They established the Draize test with the rabbit eye to predict human effects.

Skin is the largest organ in the body and skin structure and function was being elucidated from early times as for example by Gray et al[8] who studied the structure with electron microscopy. Elias[9,10] described the structure of the stratum corneum with corneocytes in multilayers in broad lamellar membranes surrounded by extracellular matrix. He and Michaels[11] first described it as like a brick and mortar wall. Histological studies revealed a basket -weave structure (e.g).[12] The lipophilic stratum corneum, gave barrier properties preventing water loss and absorbed chemicals must pass through the barrier.

In the 1960s and 70s several investigators, often dermatologists, started to apply radiolabeled chemicals to skin, often forearm or back and measured blood profile and urinary excretion of radioactivity to define dermal absorption and internal dose. For example Feldman and Maibach[13] applied hydrocortisone to different skin sites including scalp, axilla, back, and forearm on volunteers. The profile of skin absorption was more delayed to peak blood level than for other routes. They also applied a range of organic compounds to the ventral forearm for 24 h and showed penetration of the compounds with a 250-fold range and maximum absorption rate to 1,000-fold. Maibach published extensively on human in vivo absorption through the skin for many years and his work was reviewed in[14,15]. Hadgraft, who had many publications on delivery, also reviewed the early literature[16].

In vitro models of dermal absorption

A milestone change occurred when some of the seminal studies on dermal toxicology and absorption were conducted with skin in vitro. Acceptance of human volunteer exposure to chemicals was reducing and as a highly accessible organ skin lent itself to in vitro approaches. In the 1950s and 60s skin was mounted in static or flow-through diffusion cells for studying dermal absorption, as was reviewed by Ajjarapu and Maibach[17] In 1975, Franz published an important paper comparing several chemicals with a static cell to measure absorption though isolated skin.[18] The approach was to clamp skin above a receptor chamber maintained at a constant temperature and apply chemical to the skin surface. Feldman and Maibach paralleled their human in vivo data for 12 chemicals with Franz's in vitro predictions using the static cell and showed a reasonably close prediction.[18–20] The current design of the static absorption cell has not been significantly modified from the early cells.

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Other comparisons of in vivo to in vitro dermal absorption were conducted. For example, application of lindane to human forearm compared to human skin in static cell with ethanol/water as receptor[21,22] Lindane a lipophilic pesticide was slowly absorbed and eliminated in urine following metabolism. The In vivo skin surface and in vitro skin were swabbed and the stratum corneum reservoir was removed by tape stripping for analysis.

The flow through diffusion cell with tissue culture medium as receptor fluid to maintain viability of skin and support metabolism during absorption was evaluated in 1985 by Bronaugh who had been developing it for years[23] and modifications were applied by others.[24] Absorption data more closely reflected in vivo profiles than with non-viable skin. Often dermatomed skin with excess dermis removed is used to more closely reproduce in vivo absorption conditions.

Early studies took human in vivo data as the gold standard and compared in vitro predictions. There was a good relationship for a range of chemicals with differing properties. If in vitro studies are well designed and controlled they do predict absorption in vivo and were accepted by the OECD. Loss of the applied dose by sloughing off of stratum corneum in in vivo studies is not easily replicated in vitro and reservoir values must be related to chemical characteristics such as lipophilicity[25] In human volunteers dermal absorption of butoxyethanol[26,27] paralleled in vitro studies with human skin Traynor.[28]

By the early 2000's much in vitro data was being generated and there was a need to define how variable approaches were for example difference between laboratories and study design prior to establishing Guidelines. There were a number of funded consortia to evaluate in vitro approaches to ensure reliability of data and a EU dermal network was established. For example EDETOX with 12 participants addressed all areas using in vivo human studies as gold standard, and the relation to in vitro predictions with human skin and in-silico predictions.[29] A cross institution study of in vitro dermal absorption of three marker chemicals caffeine, testosterone and benzoic acid[30] is still widely quoted. Using the standardized methodology form each laboratory both static and flow through cells and human skin, significant inter-laboratory and inter-assay variation was observed. One major influence was the limited availability of suitable human skin, whether breast or abdomen with no control on age, treatment, length of storage etc. Use of a reference chemical such as caffeine with all studies was suggested. Parallel cross institution studies found similar variability e.g.[31] An eight fold range in absorption of testosterone was determined when using skin from different individuals[32] and inter-individual and intra-individual variability in human skin barrier function was shown to be high.[33]

In the early 2000s OECD having reviewed dermal absorption methodology recommended skin absorption Guidelines 428[34] which were updated in 2019 and studies following the Guidelines are accepted for regulatory purposes.[35]

Current approaches developed for cosmetics are included in SCCS Guidelines for Cosmetics in Europe following a ban on in vivo animal studies in 2015.[36] In particular currently Cosmetics Europe are appreciative of the importance of reliable reproducible in vitro dermal absorption data and have supported a recent study of the penetration of 56 cosmetic relevant chemicals through human skin following cosmetic relevant exposures.[37] The standardized OECD protocol was used with finite aqueous dose for 24 h with human skin in flow through diffusion cells. They established a database of finite dose data for evaluating future models. The authors commented that there was some variability due to outliers and donor skin variation and the reproducibility was good. They also commented that there were not major improvements on the reproducibility of historical data. Absorption data generated with in vitro models in the 70s and 80s is included in a number of databases as discussed in a later section. Historical data is effectively used in predictions and data is accepted for regulatory purposes if the study has been conducted following OECD Guidelines.

Local metabolism in skin

Local metabolism in skin during absorption has often been ignored as it is considered to have an insignificant effect on absorption of the parent molecule. However this would depend on the levels in the skin of the metabolizing enzymes involved. CYP enzymes are low but this is not so for esterases, alcohol dehydrogenases or conjugating enzymes.[38]

Early studies[39] using minced human skin showed metabolism and induction in vitro although a limitation was a rapid loss of activity of CYP enzymes as soon as skin was isolated even if stored at −80C. In 1973 Alvares[40] studied oxidative metabolism of benzopyrene by minced human skin blisters by measuring fluorescent oxidation products and induction by polyaromatic hydrocarbons. Also aldrin epoxidation to dieldrin was detected using gas chromatography with electron capture detection.[41]

There are many reviews of skin metabolism including,[42–44] Enzymes in skin have an extra hepatic profile of isoforms for both phase 1 and 2 enzymes Phase 2 enzymes which are cytosolic and microsomal are relatively high compared to CYPs which are located only in microsomes of keratinocytes with limiting access.

Hydrolysis of chemical esters applied to the skin was observed but the carboxylesterases involved were not identified as CES2 extrahepatic highest in skin and CES1 mainly hepatic till the 2000s.[45] The specificity of CES isozymes in skin has influenced the hydrolysis of paraben ester following topical application and benzoyl and butyl paraben with larger leaving group had the greater affinity for CES 2 induced by dexamethasone in skin compared to methyl and ethyl which favored CES1 and this influenced absorption.[46,47]

Cosmetics Europe have assessed the capacity of epidermal models, keratinocytes and human skin to metabolize a series of marker chemicals by phase 1 and 2 pathways.[48,49] Gotz et al raised similar issues to those highlighted in the 1980s such as lack of sensitive methodology for low levels of CYP enzymes, and influence of localization to different cell types.[50]

In human in vivo studies it is difficult to distinguish local skin metabolism from hepatic as it may be less important in influencing internal dose. PBPK requires rate constants for metabolism and uptake to cells in different organs and it is important to understand quantitative specificity of skin metabolism and compare it to liver and to identify for which molecules not considering metabolism is an issue.

The effect of local metabolism in the skin on internal dose has not been extensively considered in conjunction with flux prediction.[50–52] Further evaluation of the influence of local dermal metabolism, compared to that in the GI tract and liver, is required for bioavailability estimates and pbpk modeling for systemic blood levels. The limitation is that this can only be assessed for chemicals for which data are available. There is generally limited information available on the capacity of skin to metabolize specific chemicals so grouping and read across will need to be applied.

In the absence of experimental data prediction of skin metabolism for a chemical could feed into pbpk modeling leading

to internal dose and toxicity evaluation helped by consideration of example chemicals for which there are available toxicity data and which undergo first pass metabolism.[53] Classifying the relative bioavailability between the oral and dermal routes on a structural basis for high or low dermal absorption and high or low metabolism will improve the reliability for profiling internal/blood and organ levels of parent and metabolite for dermally applied cosmetic chemicals.[54,55]

Structure activity relationship (SAR) and dermal absorption

The most conservative case for an estimate of dermal absorption is 100% exposure. In the absence of experimental data from in vitro systems this could be used. Also, prediction in the absence of in vitro data might use a SAR algorithm. Historically, in the absence of experimental data, consideration of structure-activity relationships allowed prediction of absorption from physicochemical properties. Kroes et al proposed ranking of chemicals on the basis of J max (maximum predicted flux) to three bands of default absorption[56] However currently, in vitro data is considered in preference to a prediction and recently 50% exposure dose considered more representative than 100% has been adopted in the absence of in vitro data by SCCS.[57]

Gathering together and assessing good experimental data and curating them into datasets is important for supporting predictions. The earliest database was gathered by Flynn[58] and used by Potts and Guy.[59] The later EDETOX database gathered 50 chemicals and was made publicly available.[29,60] The Kent database added further chemicals[61] and the data were added to the RIFM and Munro databases in the COSMOS DB[62] comprising 966 chemicals.[63] Other databases have been collected for example Buist[64] and HuskinDB.[65] All databases are useful resources even though not all data is curated However it may still be necessary to generate fresh in vitro data for a specific exposure scenario.

Modeling and predictions

In the early 90s the Potts and Guy equation for predicting absorption had a major influence [59]. This was one of the earliest models which was fairly simple and transparent and still widely used today. Potts and Guy used the Flynn database of infinite dose data to validate log Kp (saturated aqueous) from log p and molecular weight. Cleek and Bunge[66] added a modification to extend to more lipophilic molecules. The Potts and Guy equation was applied to a database of fragrance chemicals.[67] There are now many models and SARs for predicting skin penetration of differing complexity using the same or similar data sets and delivering different outcomes.– all have advantages and disadvantages. Some approaches use the Potts and Guy equation and others use different SAR relationships e.g.[68,69] Recent models include MPML MechDermA with sim cyp simulator.[63]

Cosmetics Europe have recently evaluated six currently used in silico skin penetration models (using 25 model compounds applied as finite doses in non-aqueous vehicles compared in vitro absorption with split thickness human skin).[70] There was variation between models in their ability to predict penetration and distribution through the skin and uptake and efflux into cells of epidermis and dermis. The relationship to experimental data highlighted areas to improve accuracy of predictions.

When an infinite dose is applied to the skin surface a steady state flux is established and the percentage absorbed can be measured. Infinite dose exposure data gives reliable measures of lag time and steady state flux by applying the fixed fractional approach. QSARs have generally been designed using data generated with saturating doses but many dermal exposures are finite doses and it is appreciated that the QSARs do not take into account the effects of decreasing dose, evaporation, vehicle, mixtures etc. There are several approaches to explaining finite dose kinetics but the approach of Frasch et al[71] and modeling by Kasting[72] are highlighted. Their data driven approach includes Nderm and Nevap for finite volatile doses and the study addresses pentabromodiphenylether and bromopropane as examples. Availability of targeted data sets of in vitro data with evaluated methodology for actual finite exposures will help to improve applicability of future new QSARs.[73] A recent human in vivo study with sun-screen products and parallel in vitro data applied as finite and infinite doses will help NAM predictions.[74]

The Potts and Guy equation was recently used for finite doses and non-saturated non aqueous applications of cosmetic chemicals. The approach was to derive an adjusted Jmax from predicted log p taking into account the actual solubility in the vehicle formulation and the saturation solubility in this vehicle. The prediction was assessed for 22 chemicals in different formulations for which experimental data existed.[54] The ratio of experimental flux to predicted flux generally fell between 0.1 to 10 which the authors regarded as acceptable considering typical variability in experimental data, as described elsewhere in paper, and the assumptions and approximations involved in calculating flux. For regulatory purposes values greater than one which are conservative might only be regarded as acceptable. This variability indicates how evaluation of approaches like these relies heavily on good experimental data and similar approaches with new absorption datasets generated for purpose are needed.

It is now being appreciated that the Internal Dose estimated from the internal plasma level will give a more accurate prediction than existing QSAR approaches using dermal flux. Complex pbpk modeling approaches are being developed and requisite experimental data generated.[75] Cosmetics Europe are establishing a database for 200/300 chemicals used in cosmetics with plasma level and internal dose predictions.[76] They are generating in vitro skin absorption data and CaCo2 absorption and hepatocyte metabolism data. Internal Threshold of Toxicological Concern (iTTC) values have so far been derived for a number of cosmetic chemicals where dermal absorption data is available and evaluated with clinical data.[77]

The current and future in modeling

In vitro methods and structure-activity approaches are now classified as “new alternative methodologies” (NAMs) together with models. NAM approaches require databases of reliable experimental dermal absorption data and an understanding of dermal metabolism and uptake for chemicals. In vitro and in vivo measurements are needed to drive and evaluate pharmacokinetic models which must include interindividual variation in metabolism and exposure.

Read-across has been developed in many areas of toxicology and is being used more extensively as data sets expand. Collated existing data for structural analogs can be read across to help define absorption and internal exposure for a structurally related chemical. A need to define uncertainty has led to establishment of guidelines Madden 2020.

A 10 step NAM framework including read-across (RAX) for cosmetics was recently published.[78,79] This approach is moving

forward but still requires exposure and internal dose, structure and metabolite as well as toxicity data but gaps in experimental data lead to conservative prediction values being adopted. Inclusion of data from related analogs by read across extends the approach. Study of cases where there is extensive experimental data can help to increase confidence in framework predictions. The framework was applied to a case study with parabens, a homologous series of esters for which there are extensive data and incorporated both toxicokinetic and toxicodynamic data to predict a hypothetic data gap then allowing prediction of reproductive toxicity.[80] Other examples have used caffeine, for which extensive data exist, with a new framework.[81]

In the future there will be further development and refinement of predictive models but validation with data-rich chemicals will be needed for acceptance. In the absence of data, conservative approaches will be applied. Dealing with uncertainty is important before application to meaningful safety assessment. All would be helped by a new body of in vitro evidence.

What does the future hold for in vitro skin models? How will they be placed or will they be superseded?

There is a need to quantitatively combine reliable measures of dermal absorption and metabolism in skin equivalent models and for dermato-kinetics and improved experimental models to focus on skin metabolism, Prediction of the relative importance of metabolism in the skin from the chemical's structural properties including the balance between rate of penetration through skin and access to cells and enzymes would aid prediction.[54,82] Recently metabolism and absorption were combined in a predictive model of dermal uptake and the approach evaluated for an aromatic amine. Defining the role of transporters in the balance between uptake of active metabolites to target cells and export as detoxified conjugated metabolites is also important. . An early example of the importance was protection against sensitizing metabolites of dinitrochlorobenzene by export of glutathione metabolites.[83]

There is a need for greater understanding of inter-individual variability in skin permeability. Investigators have not been able to predict whether dermal absorption will be high or low for an individual skin in vitro or in vivo although variability with site on body and at the extremes of age have been demonstrated More understanding of interindividual variability might predict some of the variability in experimental measurements of absorption. Currently, for regulatory purposes, age dependent differences in skin properties are not thought to require separate approaches when defining absorption for children, or the elderly compared to adults.

Concurrent development of 2-D and 3-D cell-based models of skin using keratinocytes and fibroblasts has allowed understanding of uptake and transport into cells, local metabolism and mechanisms of dermal toxicity. Compromised skin models have been established[84] Currently developed 3-D models are often used in irritation studies where the immature stratum corneum and barrier properties are less of a problem.

Approaches to developing personalized skin equivalents using an individual's own keratinocytes have been proposed for study of skin disease but not adapted to model absorption. As skin equivalent models do not currently have a fully developed stratum corneum, prediction of dermal absorption would be an overestimate. 3D printing can now generate skin disease models for research and the cell patterning technology allows high repeatability or introduction of variability at the level of the cell. Large sheets of skin are generated for wound healing but a lack of a fully developed stratum corneum barrier still limits the approach for absorption predictions.[85]

Development of skin equivalents with improved barrier properties having overcome epidermal shrinkage is progressing.[86] It has been suggested that an increased number of stem cells among keratinocytes and Petrova obtained a functional permeability barrier in human epidermal equivalents with human epidermal stem cells and induced pluripotent cells.[87] Microengineering has generated skin on a chip systems which have been compared with diffusion systems using an artificial membrane to rank their use for ranking dermal delivery formulations.[88] Further research and development in this area is to be expected.

It is important to quantitate the intracellular concentration of absorbed chemicals and their metabolites in keratinocytes or the epidermal compartment to supply modeling. Sensitive methodology such as MALDI can locate and quantitate the chemical and metabolite in cells, e.g. studies of tofacitinib uptake in human skin.[89]

Conclusions

In this era when new predictive approaches are being developed it has been timely to reflect on the history of studies of dermal absorption and the current use of in vitro dermal absorption data generated in the past and then to consider improvement in current predictive approaches. Guidelines for generating invitro absorption data have evolved but not changed greatly, while those for QSAR and PBPK frameworks are rapidly developing. Current Guidelines for Risk Assessment as proposed by SCCS recommend use of data from a good invitro study even if not generated with exactly the same exposure scenario; rather than using a prediction. Enough good absorption data to feed models, to derive internal dose even if read across from curated databases can be used We cannot currently predict from molecular structure whether local metabolism in skin will be significant in relation to absorption, internal dose or local toxicity. Until there are many more studies with ranges of chemicals and exposure scenarios this may continue to be the case. To conclude, although there is progress with modeling dermal exposure the limitation of availability of experimental data as outlined 10–20 years ago still applies.

Conflict of interest statement

FMW is an Emeritus Professor and provides expert advice to Academia and Government

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