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RESEARCH ARTICLE

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Measurement of the penetration of 56 cosmetic relevant chemicals into and through human skin using a standardized protocol

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Abstract

OECD test guideline 428 compliant protocol using human skin was used to test the penetration of 56 cosmetic-relevant chemicals. The penetration of finite doses (10 μL/cm^2) of chemicals was measured over 24 hours. The dermal delivery (DD) (amount in the epidermis, dermis and receptor fluid [RF]) ranged between 0.03 ± 0.02 and 72.61 ± 8.89 μg/cm^2. The DD of seven chemicals was comparable with in vivo values. The DD was mainly accounted for by the amount in the RF, although there were some exceptions, particularly of low DD chemicals. While there was some variability due to cell outliers and donor variation, the overall reproducibility was very good. As six chemicals had to be applied in 100% ethanol due to low aqueous solubility, we compared the penetration of four chemicals with similar physicochemical properties applied in ethanol and phosphate-buffered saline. Of these, the DD of hydrocortisone was the same in both solvents, while the DD of propylparaben, geraniol and benzophenone was lower in ethanol. Some chemicals displayed an infinite dose kinetic profile; whereas, the cumulative absorption of others into the RF reflected the finite dosing profile, possibly due to chemical volatility, total absorption, chemical precipitation through vehicle evaporation or protein binding (or a combination of these). These investigations provide a substantial and consistent set of skin penetration data that can help improve the understanding of skin penetration, as well as improve the prediction capacity of in silico skin penetration models.

KEYWORDS

cosmetic ingredients, dataset, dermal delivery, harmonized, human skin, penetration

This collaborative work was conducted under the direction of the Cosmetics Europe Skin Bioavailability and Metabolism Task Force.

1 | INTRODUCTION

The Cosmetics Europe ADME Task Force has several projects aimed at measuring relevant parameters to help predict local and systemic bioavailability of topically exposed compounds. Endpoint measurements include solubility in different solvents (Grégoire et al., 2017); partition and diffusion coefficients (Rothe et al., 2017), metabolism (Genies et al., 2018) and skin penetration (Gerstel et al., 2016; Jacques-Jamin et al., 2017). The development of predictive in silico skin penetration models for the safety assessment of dermally applied cosmetics is needed to enable moving away from a 50% dermal bioavailability assumption (SCCS, 2018) to a more realistic one assessing the actual fraction of a systemically absorbed compound. Several mechanistic skin penetration models currently exist but the development of these models has generally been based on datasets obtained from several different publication sources, thus resulting in large heterogeneity (Chen, Lian, & Kattou, 2016; Kasting & Miller, 2006; Miller & Kasting, 2010; Polak et al., 2012). For example, existing databases report studies that tested the skin penetration of chemicals using different vehicles, species, skin thicknesses and durations. Moreover, the most used database (i.e., Flynn database; Flynn, 1990) report permeability coefficient measurements obtained in an infinite dose, which is not reflective of realistic consumer exposure. This makes it much more difficult to correlate skin penetration with chemical characteristics, which is needed for the development of in silico skin penetration models. To this end, we have generated a comprehensive in vitro bioavailability dataset for 56 chemicals using an OECD Test Guideline compliant (OECD, 2004) standardized protocol for each assay.

The chemicals for this project were specifically selected to be relevant to the cosmetics industry, including positive and negative reference chemicals for genotoxicity and/or skin sensitization assays. A complete list of the chemicals, together with their use, known toxicity and physicochemical properties are listed in Table S1 (see Supporting Information). Of the 56 chemicals tested, 33 are, or have been, used as ingredients in cosmetics (most are in current use but three are restricted and two are banned due to skin toxicity). Some of the chemicals have been tested in the Cosmetics Europe genotoxicity and/or skin sensitization assays as positive controls: 29 are skin sensitizers (including four genotoxic sensitizers) and an additional three are genotoxins. Finally, to compare the outcome of the in vitro assays with in vivo data, we have included five chemicals that have extensive human in vivo data (hydrocortisone, testosterone, ibuprofen, benzoic acid and caffeine). Additional selection criteria included a logP between −1 and 4, a molecular weight between 100 and 500, no mixtures and, as far as possible, a low volatility.

Until now, there have been no other studies that have generated such a comprehensive database of results from standardized assays using a finite dose for such a large number of chemicals. This set of data will help improve the understanding of skin penetration of chemicals with varying physicochemical properties, as well as improve the prediction capacity of in silico skin penetration models.

2 | MATERIALS AND METHODS

2.1 | Chemicals

All chemicals were exclusively tested as ^{14}C-radiolabeled solutions. The radiolabeled chemicals were used as tracers and were mixed with label-free chemicals to achieve the final concentrations with a radiolabel concentration of 0.30 μCi/10 μL.

Radiolabeled chemicals were from ARC Inc., Selcia Ltd., Quotient Bioresearch or from Moravec Inc. The label-free chemicals were from Sigma-Aldrich, Chemos GmbH or Sensient Cosmetic Technologies. All other chemicals and solutions used were from Sigma-Aldrich. A full list of the suppliers of label-free and radiolabeled chemicals (and their specific activity) is shown in Table S2 (see Supporting Information).

2.2 | Solvents

There were 56 chemicals tested in these studies, of which 49 were applied in phosphate buffered saline (PBS) and six were tested in 100% ethanol (2-acetyl aminofluorene, 4-bromophenyl isocyanate, naphthalene, testosterone, tetramethyl thiuram disulfide and triclosan) and one was tested in acetone (benzyl bromide). Four chemicals were tested in PBS or in 100% ethanol: benzophenone, geraniol, hydrocortisone and propylparaben, i.e., two sets of experiments per chemical testing the effect of solvent on their cutaneous distribution. In addition, propylparaben was tested in two separate experiments to explore intralaboratory reproducibility.

The target solvent for the penetration studies was PBS because we wanted to avoid solvents, e.g., ethanol and dimethyl sulfoxide, which may alter the lipid structure in the skin and thus the penetration characteristics. In addition, the majority of currently available in silico models have been trained on skin penetration data generated using aqueous vehicle and either lack the capability to include non-aqueous vehicles (with exceptions: Gregoire et al., 2009; Riviere & Brooks, 2007) or have not been fully evaluated for this purpose due to a lack of data (Dancik, Miller, Jaworska, & Kasting, 2013; Selzer et al., 2013) and therefore require the solvent to be aqueous. The solvent used for each chemical is listed in column O of Table S3 (see Supporting Information). PBS was considered a suitable solvent because it is aqueous and has a buffering capacity to maintain the pH of the dosing solution near to neutral (6.0-7.5). PBS with a phosphate concentration of 0.01 M, pH 7.4 was used for 31 chemicals. When the chemical caused the pH of the application solution to deviate outside the range pH 6-7, a higher phosphate concentration of 0.1 M was used (13 chemicals). The exception to this was dimethyl fumarate, which was more stable when the pH was adjusted to pH 5. Some chemicals are prone to oxidation in aqueous solutions; therefore, in these cases, antioxidants (3% ascorbic acid and 0.4% sodium sulfite) were added (four chemicals with 0.01 M PBS and two chemicals with 0.1 M PBS). Six chemicals (2-acetyl aminofluorene [2-AAF], 4-bromophenyl isocyanate [4-BPI], naphthalene, testosterone, triclosan and tetramethyl thiuram disulfide) were not sufficiently

soluble in water to be able to reach the required level of radioactivity and were therefore dissolved in 100% ethanol. One chemical (benzyl bromide) was dissolved in 100% acetone, as it was not chemically stable in PBS or ethanol.

2.3 | Dose setting

Finite doses of each chemical were applied to the skin. It was not possible to apply exactly the same dose in mg/mL of each chemical because they differed widely in their solubility in water. Therefore, the concentrations of dosing solutions were calculated in a systematic fashion. First, the maximum amount of an infinite dose penetrating the skin was predicted based on logP, molecular weight (mw) and maximum solubility in water (S_W), using the equation of Kroes et al. (2007). These equations were adapted from the Potts and Guy relationship to enlarge the applicability domain to lipophilic compounds (Potts & Guy, 1992).

$$Log\,Kp = -2.7 + 0.71\,x\,logP - 0.0061\,x\,mw$$
$$Kp_{corr}(cm/h) = \frac{Kp}{1 + Kp\,x\sqrt{mw}/2.6}$$
$$J_{max} = Kp_{corr}\,x\,S_W(mg/ml)$$
$$Amount\ absorbed\,(mg) = J_{max}\,x\,A\,x\,t_{exp}\,x\,DS,$$

where the area of skin (A) (cm^2) = 1; time of exposure (t_{exp}) (hours) = 24; degree of saturation (DS) = 1; Kp = permeability coefficient; Kp_{corr} = corrected Kp (necessary to take into account the contribution of the viable epidermis and dermis to the permeation process for very lipophilic chemicals); J_{max} = maximal flux.

Secondly, the maximum amount absorbed was divided by 100 to extrapolate to dose applied to the skin that could be considered finite. If the calculated finite dose was not practically possible due to the value being near the limit of its aqueous solubility, a dose equal to 60% of the maximal solubility in water was taken (the solubilities of 50 chemicals in water is reported by Grégoire et al., 2017), provided this resulted in sufficient radioactivity (0.3 μCi/10 μL). For chemicals that were readily soluble but the specific activity of the radiolabel was low, the calculated finite does did not result in sufficient radioactivity, and the concentration was increased accordingly (to result in 0.3 μCi/10 μL).

2.4 | Dosing solution checks

The homogeneity of the dosing solution was controlled with six aliquots (even distribution on the upper, middle and lower layers of the solution). The homogeneity, expressed as the %CV of the distribution in the solution layers for each chemical is shown in Table S3 (see Supporting Information). If the %CV was >5%, a fresh solution was prepared. There was only one chemical with a %CV higher than 5%, namely naphthalene; however, this value was 7% and was considered acceptable considering it was very volatile.

The stability of the chemical in the solution over 24 hours at 32°C was tested by radio-high-performance liquid chromatography, confirming the presence of a single peak at the retention time of the parent chemical. The radiopurities (measured by radio-high-performance liquid chromatography) of all dosing solutions before and after a 24-hour incubation at 32°C were ≥90%, with the exception of 4-tolunitrile and ethylhexyl acrylate, which were 81% and 88% pure after a 24-hour incubation.

2.5 | Skin tissue

Abdominal human skin was ethically obtained with consent for research from 248 Caucasian donors undergoing surgery from commercial suppliers and in accordance with French laws (Banque de Tissu Lyon, Biopredic International, Saint-Grégoire and Alphenyx). Nineteen donors were male (aged between 24 and 57 years of age) and 229 were female (aged between 22 and 77 years of age). The skin was devoid of obvious damage, disease or stretch marks. The fresh skin was frozen within 24 hours of shipment and stored at −20°C for 8-12 weeks. This storage duration was shown not to affect the penetration of three chemically and metabolically stable chemicals (Jacques-Jamin et al., 2017) and was therefore used as a standard storage duration for all skin used in these studies. The skin disks were thawed at room temperature for 30-45 min and dabbed dry using cellulose swabs. For each chemical, three replicate discs from each of four donors were used. Human skin was dermatomed to a thickness of 400 ± 50 μm.

2.6 | Skin penetration experiment

An all-in-one mounted system, the Permegear Fraction Collector FC33 (from Analysesysteme), was used for these studies. This includes an automated fraction collector with in-line cells; a warmer and tubing guide; a recirculating water bath (SES-ED5-Heater/Circulator); a set of seven in-line cells; a peristaltic pump and a distribution manifold. Flow-through cells with a 1-cm^2 application area were used. The receptor compartment of the diffusion cell was filled with RF (0.9% NaCl in water, supplemented with 1% [w/v] bovine serum albumin and 0.05% [v/v] gentamycin sulfate). Flow rate was adjusted to 1 mL/h. The solubility of each chemical was tested in the RF to confirm sink conditions. Skin discs were placed on to the receptor compartment of a diffusion cell (made of polychlorotrifluoroethylene), and the donor compartment was then placed on to the skin, and both compartments were tightly clamped. The clamps were stainless steel, and the glass between the two compartments was polycarbonate. All experiments were conducted using non-occluded conditions. The temperature of the diffusion chamber and skin was measured at the start of the experiment and maintained throughout the assay at 32 ± 2°C. Once mounted, the diffusion cells were allowed to equilibrate for 1 hour. After equilibration, the skin integrity was confirmed before application of chemicals by measuring

the transepidermal water loss (TEWL) using a Tewameter® TM300 and probe from Monaderm (recorded in columns Q and R in Table S3; see Supporting Information). Only skin discs with a TEWL between 0.7 and 5 g/m^2/h were used in the experiments (based on the historical measurements of the testing laboratory).

A volume of 10 μL/cm^2 of appropriate solvent containing each chemical was applied to the surface of the skin (1 cm^2) using a positive displacement pipette and spread (without pressure) to ensure an even distribution of the solution. The dose of each chemical applied is provided in Table S3 (see Supporting Information).

The RF was sampled 1 hour before dosing and then after dosing at 30 minutes, 1, 2, 4, 8, 16 and 24 hours. After 24 hours, the skin surface was washed with 0.5 mL water containing 10% Tween 80® using a cotton bud and gentle rubbing for 30 seconds. The skin was then washed seven times with 0.5 mL water and then carefully dried using cotton buds. The skin wash solutions, cotton buds and tips were collected for analysis. After the skin wash, the cell system was dismantled. The donor and receptor compartments were placed in flasks and 10 mL and 40 mL ethanol was added, respectively, and the closed flasks were shaken at least overnight. The skin was tape stripped using adhesive scotch tape Magic 3M, with a weight of 150 g/cm^2 placed on top of the Scotch tape for 10 seconds before removal. A maximum of 20 strips were taken and pooled in a vial as follows: strips 1-2, 3-8, 9-14 and 15-20. For each skin disc, the number of tape strips taken until the epidermis was reached was recorded and mean ± SD values are listed in column X and Y, respectively, in Table S3 (see Supporting Information). Soluene®-350 (3 mL) was added and the vial was placed at about 60°C until the stratum corneum (SC) samples were dissolved. The epidermis and partial dermis were separated by heating at 60°C for 40 seconds and using a scalpel blade. Soluene®-350 (3 mL) was added and the vials were heated at about 60°C until the samples were dissolved. Appropriate volumes of Ultima Gold scintillation liquid were added to each of the samples, which were then analyzed for radioactivity using a scintillation counter.

2.7 | Data analysis

The dermal delivery (DD) was calculated as the total amount present within epidermis (without the amount in the total SC strips), dermis and RF. The DD calculation assumes no residual SC is present on the epidermis. When the amount in the final strips 15-20 were compared with the DD, the amount was >2% of the DD for 50 experiments, between 2% and 10% for nine experiments, 11% for hydrocortisone in PBS and 25% for 4-BPI. Therefore, for the majority of the experiments, the DD is only likely to be overestimated by <2%.

Statistical analyses were conducted using Instat (Graph-Pad software). The statistic model used took into account donor effect as a random variable and a fixed effect associated to the vehicle. This allowed an estimation of the difference sources of variability, i.e., inter- and intra-donor and intra-vehicle). Considering the low number of replicates (n = 12), the variability of the data should not follow a normal distribution; therefore, the statistical model used was a non-parametric method. The relevance of the comparison was evaluated according to the size of the effect (i.e., very small, small, moderate, strong or very strong). The resulting P-value was adjusted using the Bonferroni method, where a statistical significance was reached if $P < .05$.

3 | RESULTS

3.1 | Solvent effects on cutaneous distribution

As six of the chemicals were dissolved in ethanol due to insufficient solubility in PBS (2-AAF, 4-BPI, naphthalene, testosterone, tetramethyl thiuram disulfide and triclosan), we investigated how this may affect the cutaneous distribution of the chemicals. Therefore, four chemicals were selected based on their sufficient solubility in ethanol and PBS and tested in both solvents. These were benzophenone, geraniol, hydrocortisone and propylparaben, which were selected to match the physicochemical properties of the compounds tested only in ethanol. The distribution of hydrocortisone in the different compartments was very similar when it was applied in PBS and ethanol (Figure 1A), with only moderate, albeit statistically significantly, differences in the skin compartments and DD. Likewise, the cumulative amount of hydrocortisone in the RF over time was equivalent in PBS and ethanol (Figure 1B). By contrast, the distribution and kinetics of the other three chemicals were strongly or very strongly affected by using ethanol instead as PBS as the solvent (Figure 1C-H). The main effect of ethanol was to increase the amount of chemical recovered in the skin wash and a concomitant decrease in the amount in the RF. While the amounts of propylparaben and geraniol in the epidermis and dermis were unaffected or only moderately affected by using ethanol as the solvent, there was relatively more benzophenone in the epidermis and dermis after application in ethanol than in PBS (Figure 1C). The application of benzophenone, propylparaben and geraniol in ethanol tended to result in a slower rate of appearance in the RF. This was particularly apparent for propylparaben, which had a hyperbolic profile in PBS and a linear profile in ethanol (Figure 1F).

3.2 | Dermal delivery

Figure 2 shows the range of DD values measured for the 56 chemicals and the associated doses applied in μg/cm^2. The full dataset for the DD, cutaneous distribution and mass balance of each chemical is shown in Table S3 (see Supporting Information). These were evenly ranged between 0.03 ± 0.02 (4-BPI) and 72.6 ± 8.9 μg/cm^2 (resorcinol). The doses were established based on the solubility of the compounds, resulting in the majority of chemicals applied ≤10 μg/cm^2 (42 of 56 chemicals), with eight doses between 12 and 25 μg/cm^2 and six doses between 47 and 98 μg/cm^2. There was a general trend between the dose applied and the resulting DD expressed as μg/cm^2 (R^2 = 0.47) but not when expressed as the percentage of the applied

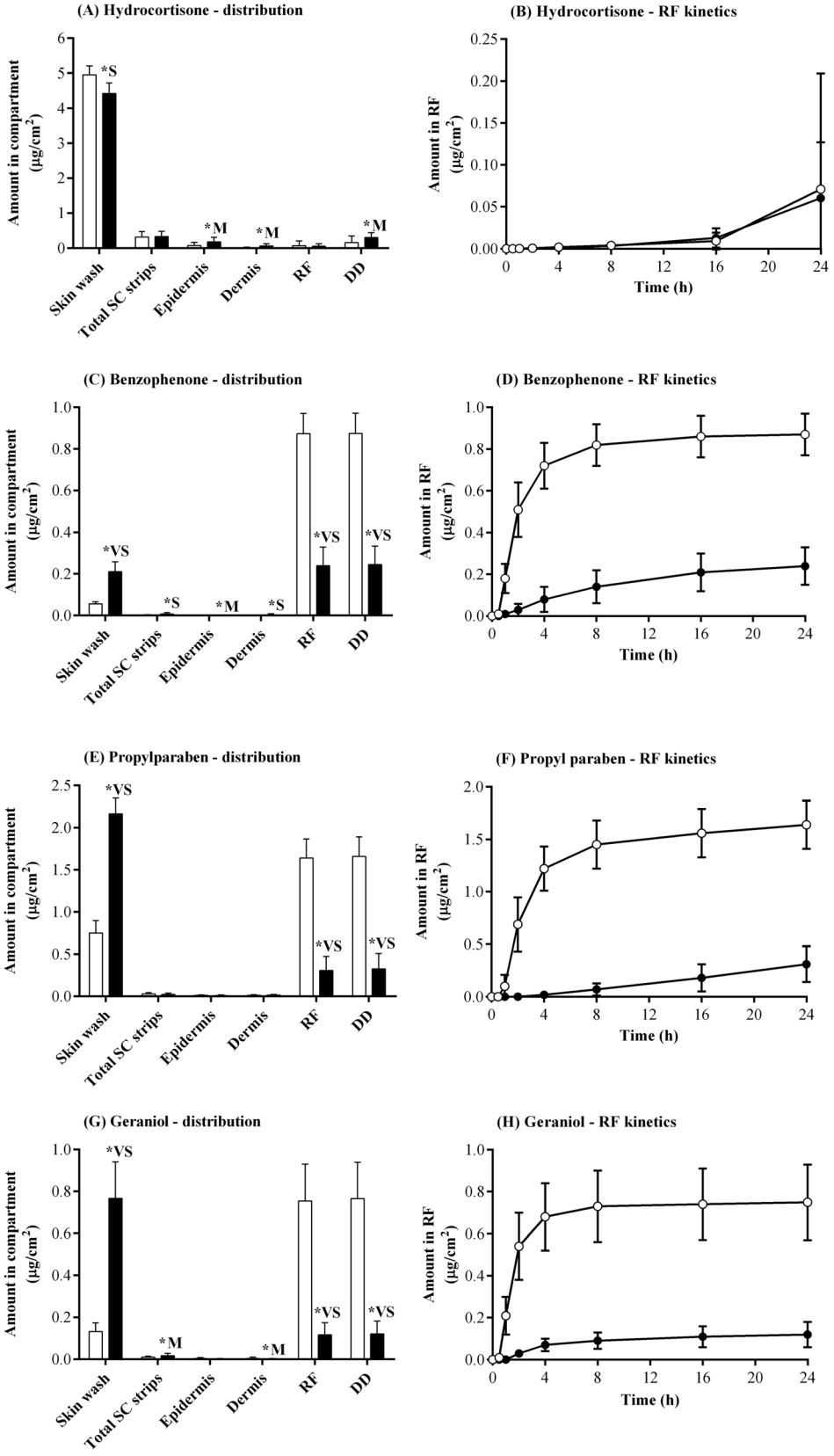

FIGURE 1 Cutaneous distribution and RF kinetics of chemicals after topical application in phosphate-buffered saline (white bars in distribution graphs or white circles in RF kinetics) and 100% ethanol (black bars in distribution graphs or black circles in RF kinetics graphs). A, B, Hydrocortisone. C, D, Benzophenone. E, F, Propylparaben. G,H, geraniol. Values are mean ± SD of three skin discs from four donors. Significant differences, where $P < .05$, are denoted with an asterisk and the size of the effect is as M, S or VS. DD, dermal delivery; M, moderate; RF, receptor fluid; S, strong; SC, stratum corneum; VS, very strong

dose. There was a linear correlation between the DD and the amount in the RF (R^2 of 0.99) (Figure 3), indicating that the amount in the RF accounted for the DD for most chemicals, with no accumulation in the SC, epidermis or dermis. However, there were exceptions to this, for example: (a) chemicals tested in ethanol, e.g., 4-BPI (RF amount represented 15% of the DD) or in PBS, e.g., 2-amino-3-methylimidazo(4,5-f)quinolone (IQ; RF amount was 6.9% of DD); (b) chemicals with low DD, e.g., diethanolamine (RF amount was 17% of

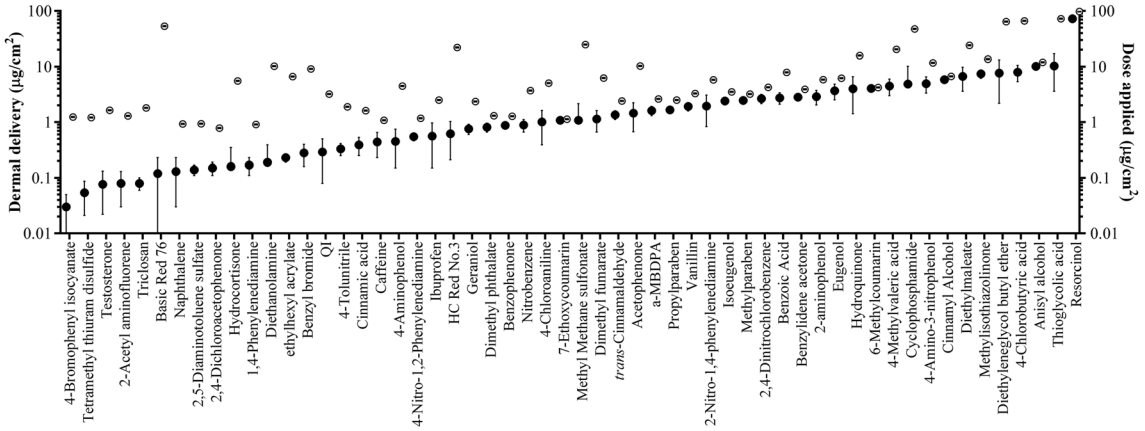

FIGURE 2 Dermal delivery values of 56 chemicals. Values for dermal delivery (closed circles) are mean ± SD of three skin discs from four donors. Doses applied (open circles) are in µg/cm^2. Alpha-MBDPA = alpha-methyl-1,3-benzodioxole-5-propionaldehyde; IQ = 2-amino-3-methylimidazo[4,5-f]quinolone

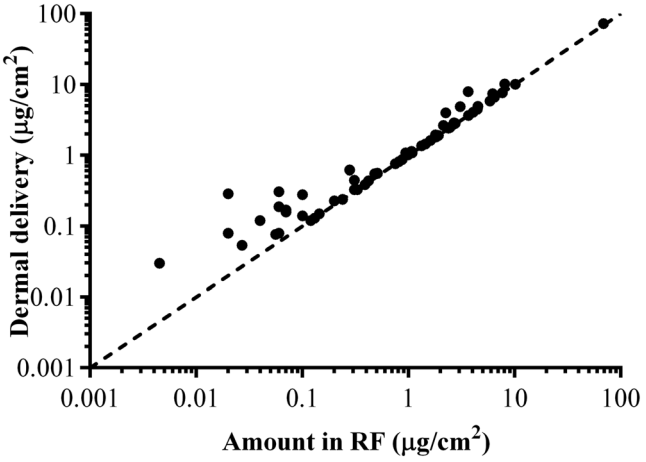

FIGURE 3 Correlation of the amount of chemical in the RF with its dermal delivery. Values are mean ± SD of three skin discs from four donors. RF, receptor fluid

DD); or (c) chemicals with higher DD, e.g., 1,4-phenylenediamine (RF amount was 44% of DD).

3.3 | Receptor fluid kinetics

The data for the receptor kinetics for all 56 chemicals are shown in Table S3 (see Supporting Information). Excluding chemicals tested in ethanol (hydroquinone, benzophenone, geraniol and propylparaben), the cumulative amount detected in the RF for the majority (42 of 56) resembled a hyperbolic profile, with varying lag times (t_{lag}). The cumulative amount of 31 chemicals reached a plateau long before the termination of the experiment at 24 hours (between 2 and 8 hours). For finite doses, the observation of a plateau indicates that a chemical cannot penetrate the skin further due to one or more reasons: (a) applied dose has been depleted due to all the chemical being absorbed; (b) chemical evaporated before it could be absorbed; (c) chemical has precipitated on the surface of the skin due to solvent evaporation; or (d) covalent binding related to chemical reactivity, thus preventing further penetration. Figure 4A shows the RF kinetics for 6-methylcoumarin as an example of donor compartment depletion. The absorption of this chemical was very rapid, such that almost the entire applied dose was present in the RF after 2-4 hours. By the end of the incubation, very little of the chemical was present in the skin wash, total SC tape strips or skin layers (Figure 4A, distribution). Figure 4B shows an example (4-tolunitrile) of a chemical that was volatile and with a hyperbolic kinetic profile. The majority of the applied dose had entered the RF by 4 hours. In contrast to 6-methylcoumarin, the depletion of donor compartment was likely to be due to the evaporation from the skin surface, as the mass balance was only 20% of the applied dose and very little of the applied dose was recovered in the skin wash. Figure 4C shows the RF kinetics for Basic Red 76 as an example of a chemical that may precipitate on the skin surface causing it to have a hyperbolic kinetic profile. It has a very low DD (0.12 ± 0.11 µg/cm^2, 0.2% ± 0.2% of the applied dose), which was not due to volatility (the mass balance was 97% of the applied dose). Unlike other chemicals with large amounts of the applied dose recovered in the skin wash (Figure 4D-F), the RF kinetics for Basic Red 76 was not linear over time and reached a plateau after about 8 hours, suggesting that penetration into the skin had stopped.

Sixteen chemicals exhibited near-linear RF kinetics, with the amount in the RF continuing to increase with time, without reaching a plateau. Notably, eight of the 16 chemicals were applied in ethanol (hydrocortisone and propylparaben [also tested in PBS, see above section 3.1], naphthalene, 2-AAF, tetramethyl thiuram disulfide, testosterone, triclosan and 4-BPI). Notably, at 32°C, once the solvent has evaporated, all of these chemicals would be solids or on the border of solid/liquid state (the melting point of 4-BPI is 31°C). For all except one of these chemicals, the percentage of the applied amount remaining on the surface (and recovered in the skin wash) was very high (58%-86% of the

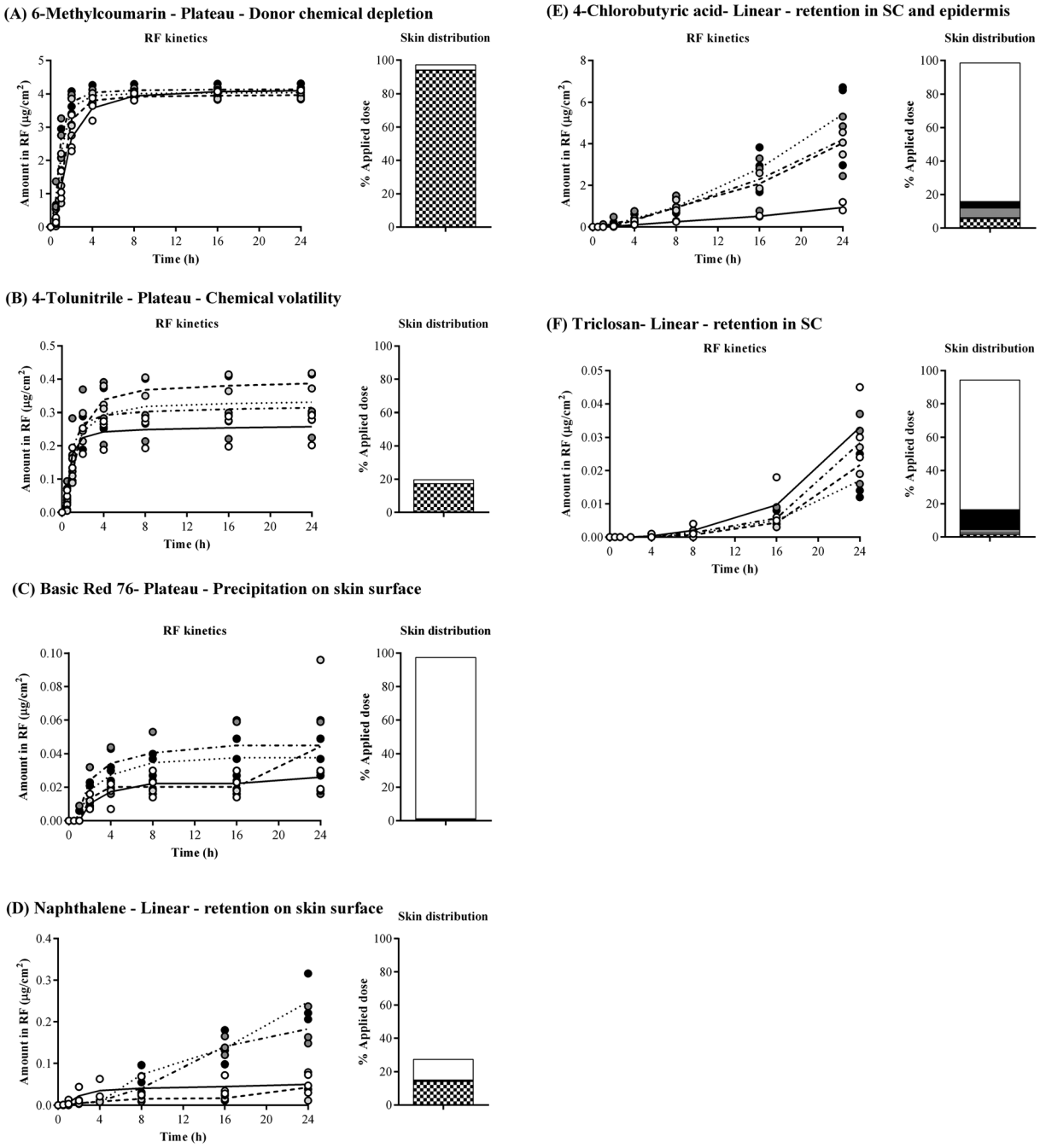

FIGURE 4 Example profiles of cumulative amounts of chemicals in the RF and cutaneous distribution of chemicals tested in skin penetration assays. A, 6-Methylcoumarin. B, 4-Tolunitrile. C, Basic Red 76. D, Naphthalene. E, 4-Chlorobutyric acid. F, Triclosan. For RF kinetics: white circles = donor 1; light grey circles = donor 2; dark grey circles = donor 3; black circles = donor 4. For distribution: white bars = skin wash, black bars = SC, dark grey bars = epidermis, light grey bars = dermis, checked bars = RF. RF, receptor fluid; SC, stratum corneum

applied dose). The exception was naphthalene, which was volatile (mass balance was 27% of the applied dose); therefore, the amount recovered in the skin wash (14% of the applied dose) was still approximately half of the recovered amount (Figure 4D). 4-Chlorobutyric acid was applied in PBS; however, it penetrated the skin slowly, with a t_{lag} of 3 hours, possibly due to retention in the SC and epidermis (3.9% and 5.7% of the applied dose, respectively, Figure 4E). These amounts were both higher than the median values for these layers for all 56 chemicals tested: 1.5% and 0.8% of the applied dose in the total SC tape strips and epidermis, respectively. The retention of the chemical in the total SC tape strips and epidermis was also a characteristic of other chemicals with slow or linear RF kinetics, most notably for triclosan, IQ and 4-BPI, of which 12%, 16% and 17% of the applied dose was recovered in the total SC tape strips. The RF kinetics and distribution of triclosan is shown in Figure 4F.

3.4 | Data variability

The reproducibility of the measurements for each chemical was very good (reflected in the small error bars shown in Figure 2 and low SD values shown in Table S3; see Supporting Information). One chemical, propylparaben, was tested in two separate experiments and the amounts in each compartment were comparable (the results for these are shown in Table S3; see Supporting Information). The variability of the data was partially correlated with the DD, such that chemicals with a DD >45% tended to have a %CV of <30%. Four main reasons for variability were: (a) low penetration, e.g., basic Red 76 with a DD of 0.22% ± 0.21% of the applied dose; (b) experiments with 1-2 data points that differed from the remaining 10-11 at one time point, e.g., hydrocortisone (Figure 5A); (c) chemicals with different absorption profiles in skin from different donor but with good replicate reproducibility, e.g., cinnamaldehyde (Figure 5B); and (d) chemicals with different absorption profiles in skin from different donor but with low replicate reproducibility, e.g., diethyleneglycol butyl ether (Figure 5C).

3.5 | In vitro versus in vivo dermal delivery

The DDs of seven chemicals tested in these studies were compared with values reported in the literature in in vivo human studies (Table 2). Where possible, values for in vivo DD were for similar applied doses, similar solvents and for exposure durations of at least 24 hours. Despite the difference in solvents used, the in vitro and in vivo DD values were remarkably similar and were within a factor of 2 for five chemicals. The greatest difference was observed for hydrocortisone; however, it was still within one order of magnitude. There was a 12-fold high absorption of nitrobenzene in the in vitro assays compared with the in vivo estimation; however, this is a volatile chemical, which is less likely to be lost in a 1-cm^2 skin disc in a diffusion cell in our studies than on a 13-cm^2 area of the forearm of human volunteers. Feldmann and Maibach (1970) did not particularly refer to the volatility of nitrobenzene but did suggest that there was a loss of some of the chemical from the skin surface during exposure. Overall, the ranking of DD was the same in vitro as in vivo, such that hydrocortisone, testosterone and nitrobenzene were poorly absorbed, and caffeine was relatively well absorbed.

4 | DISCUSSION

The penetration of 56 chemicals into and through human skin was tested using the same OECD test guideline compliant protocol. The suitability of this method to predict in vivo skin penetration accurately was supported by the good concordance between the DDs of seven chemicals measured in our in vitro studies and values reported in the literature in in vivo human studies. The demonstration of a good vivo-vitro correlation is important as under- or overestimations may often be critical for human exposure. The impact will be different depending on the intended use of the chemical. For cosmetic ingredients, it is more conservative to overestimate exposure in in vitro assays because this assumes a worst-case scenario for the calculation of the margin of safety. For topically applied pharmaceuticals,

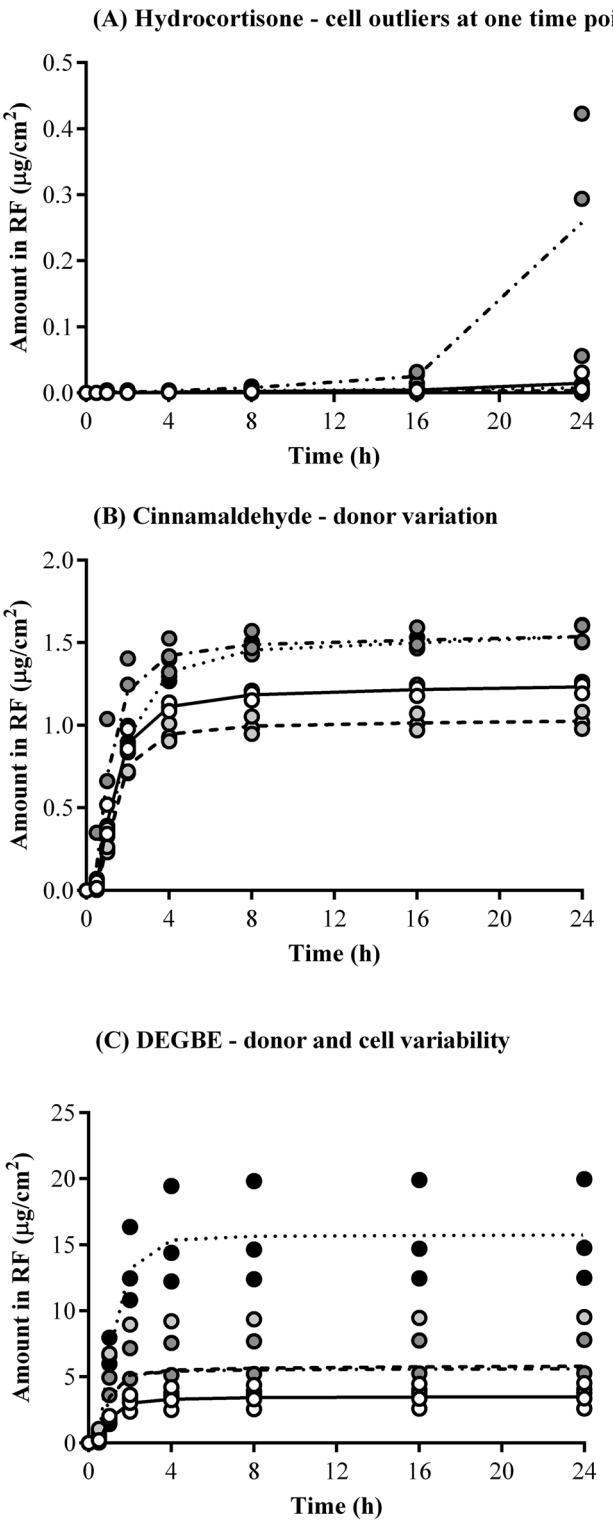

FIGURE 5 Examples of sources of variation in RF kinetics. A, Outliers at one time point. B, Donor variation. C, Donor and skin cell variability. White circles = donor 1; light grey circles = donor 2; dark grey circles = donor 3; black circles = donor 4. RF, receptor fluid

an overestimation in vitro can mean the local therapeutic concentrations are not reached in vivo and the drug fails to have efficacy. The highest vivo-vitro correlations are achieved when relevant exposure scenarios are mimicked in the in vitro test, e.g., same formulation/solvent, dose, exposure duration and anatomical site (Lehman, Raney, & Franz, 2011).

There was an even spread of values for DD, which were not correlated to the dose applied or the integrity of the skin (according to TEWL values). The even distribution of DD is an advantage when making more in-depth analyses of these data, as they are not biased towards high or low penetration chemicals. Based on the linear correlation, DD was mostly accounted for by the amount in the RF; however, there were some exceptions for chemicals with lower DD, e.g., IQ, 4-BPI, diethanolamine and higher DD, e.g., 1,4-phenylenediamine, which had accumulated in the skin layers. While 4-BPI, 1,4-phenylenediamine react extensively with peptides in the direct peptide reactivity assay (DPRA) (data not shown) and could be expected to remain in the skin layers, there were no obvious physicochemical properties that could explain why diethanolamine, IQ and other chemicals accumulated in the skin layers, which may warrant more investigations.

As these penetration assays were conducted using frozen skin, conversion of the parent to metabolites via metabolizing enzymes was not expected. Furthermore, analysis of the dosing solutions confirmed that all chemicals were of sufficient purity and were chemically stable over the 24 hours of the experiment. Therefore, the penetration values reported could be assumed to be of the parent and not of metabolites or non-enzymatic breakdown products. It was not practically possible to measure the purity of the radiolabeled chemical in the RF for such a large number of chemicals; therefore, the possibility that some chemical degradation occurred on or in the skin cannot be ruled out.

The values for DD were reproducible within an experiment, as well as across experiments (as demonstrated using propylparaben). This intralaboratory reproducibility is in accordance with others who demonstrated a 10% variability between flux values for methylparaben generated by different operators within the same laboratory (Chilcott et al., 2005). The DD of some chemicals was affected by donor differences, which emphasizes the importance of testing multiple donors to incorporate such differences into the safety assessment of topically exposed chemicals. In the interlaboratory comparison study reported by van de Sandt et al. (van de Sandt et al., 2004), the variability of the absorption of testosterone was attributed by the authors to differences in skin thickness; however, we used skin discs of similar thicknesses (384 ± 28 μm, %CV of 7%) with DD values that varied by 72% (4.7% ± 3.4% of the applied dose).

The only difference in the protocol for six chemicals (2-AAF, 4-BPI, naphthalene, testosterone, tetramethyl thiuram disulfide and triclosan) was that they were applied only in ethanol rather than the target solvent of PBS (an additional chemical, benzyl bromide, was dissolved in acetone). Therefore, four additional chemicals with similar physicochemical properties were tested in 100% ethanol and in PBS to assess how this would impact the penetration; these were hydrocortisone, benzophenone, geraniol and propylparaben. As solvents such as ethanol and acetone are described as skin penetration enhancers (Williams & Barry, 2004), it was expected that the chemicals would have a higher DD when they were applied in ethanol compared with PBS, particularly as they were more highly soluble in the former solvent (Table 1). However, this was not the case; the DD of three of the four chemicals was markedly decreased. There are several reasons for this observation. First, the amount of ethanol relative to the SC might not be sufficient to cause disruption of SC (the higher the relative amount of ethanol applied to the skin, the greater the disruption and thus the greater

TABLE 1 Physicochemical properties of chemicals tested in PBS and/or ethanol or acetone

			Solubility (mg/mL) (saturation degree)			
	logP	Molecular weight	Water	Ethanol	Melting point (°C)	Vapor pressure (mmHg)
Chemicals tested in PBS and ethanol						
Hydrocortisone	1.61	362.5	0.97 (0.60)	37.7 (0.015)	219	1.21×10^{-13}
Benzophenone	3.18	182.1	0.22 (0.59)	70.6 (0.0018)	48	0.00193
Propylparaben	3.04	180.2	0.52 (0.48)	460 (0.00054)	97	0.00031
Geraniol	3.56	152.2	0.35 (0.60)	151 (0.0014)	<15°C	0.03
Chemicals tested in ethanol only						
2-AAF	3.12	223.3	0.004	9.72 (0.011)	169	2.87×10^{-5}
4-BPI	3.48	198.0	0.035	11.4 (0.0088)	31	0.096
Naphthalene	3.3	128.2	0.03	320 (0.00034)	80	8.5×10^{-2}
Testosterone	3.32	288.4	0.13	106 (0.0014)	155	2.23×10^{-8}
Triclosan	4.76	289.5	0.04	468 (0.004)	56	4.65×10^{-6}
Thiram	1.73	240.4	0.12	1.52 (0.079)	156	1.733×10^{-5}
Chemical tested in acetone only						
Benzyl bromide	2.92	171.0	7.32	444 (0.0022)	−2	0.343

the enhancement of penetration of the test chemical). Secondly, differences in penetration could be attributed to the rate of evaporation of the solvent. Once a chemical is applied to the skin surface, the solvent can evaporate, and, as it does so, it increases the solute concentration, reaching a point at which the chemical precipitates, thus preventing it from entering the SC. The rate of evaporation of ethanol is higher than water; therefore, chemicals dissolved in ethanol are generally more likely to precipitate than when they are dissolved in aqueous solution. The three chemicals affected by ethanol all have much higher logP values and lower

TABLE 2 Comparison of the in vitro and in vivo dermal delivery of applied topically chemicals

	In vitro		In vivo		
Chemical	Dose and solvent	Dermal delivery% applied dose (μg/cm^2)	Dose, solvent, duration	Dermal delivery% applied dose	Reference
Hydrocortisone	5.53 μg/cm^2 in PBS	2.9 ± 3.4 (0.16 ± 0.19)	4 μg/cm^2 in acetone, 5 days	0.2	Wester and Maibach (1976)
	5.41 μg/cm^2 in EtOH	5.7 ± 2.5 (0.31 ± 0.14)	4 μg/cm^2 in acetone, 5 days	1.9 ± 1.6	Feldmann and Maibach (1969)
			13.33 μg/cm^2 in acetone, 7 days	0.42	Melendres, Bucks, Camel, Wester, and Maibach (1992)
			40 μg/cm^2 in acetone, 7 days	0.35	
Testosterone	1.64 μg/cm^2 in EtOH	4.7 ± 3.4 (0.077 ± 0.055)	3 μg/cm^2 in acetone, 5 days	6	Wester and Maibach (1976)
			4 μg/cm^2 in acetone, 5 days	18.1 ± 10.1 (unshaved)	Wester and Maibach (1975)
				19.0 ± 10.6 (shaved)	
			4 μg/cm^2 in acetone, 7 days	18 ± 8.6	Bucks, McMaster, Maibach, and Guy (1988)
			4 μg/cm^2 in acetone, 5 days	13 ± 3	Feldmann and Maibach (1969)
			4 μg/cm^2 in acetone, 5 days	14	Bartek, LaBudde, and Maibach (1972)
			1% hydroalcoholic gel, 24 h	9-14	Swerdloff et al. (2000)
Ibuprofen	2.51 μg/cm^2 in PBS	22 ± 16 (0.56 ± 0.41)	1.25 mg/cm^2 gel, 24 h	22 ± 12*	Kleinbloesem, Ouwerkerk, Spitznagel, Wilkinson, and Kaiser (1995)
			200 mg patch, 24 h	16	Lewis, Connolly, and Bhatt (2018)
Benzoic acid	7.92 μg/cm^2 in PBS	35 ± 7.9 (2.74 ± 0.64)	3 μg/cm^2 in methanol, 5 days	35	Wester and Maibach (1976)
			4 μg/cm^2 in acetone, 5 days	43	Feldmann and Maibach (1970)
Caffeine	1.08 μg/cm^2 in PBS	41 ± 20 (0.44 ± 0.21)	50 μg/cm^2 in EtOH/PG, 24 h	57	Liu et al. (2011)
			4 μg/cm^2 in EtOH/acetone	22	Franz (1978)
			60 μg/cm^2 in EG gel	41	Bronaugh and Franz (1986)
			4 μg/cm^2 in acetone, 5 days	48	Feldmann and Maibach (1970)
DNCB	4.26 μg/cm^2 in PBS	62.6 ± 12.0 (2.66 ± 0.51)	4 μg/cm^2 in acetone, 5 days	53	Feldmann and Maibach (1970)
Nitrobenzene	3.70 μg/cm^2 in PBS	23.9 ± 5.61	4 μg/cm^2 in acetone, 5 days	1.5 ± 0.8	Feldmann and Maibach (1970)

DNCB, 2,4-dinitrochlorobenzene; EG gel, ethylene glycol gel; EtOH, ethanol; PG, propylene glycol.

*Bioavailability compared with oral administration, which is complete and rapid.

In vivo human dermal delivery is assumed the same as bioavailability measured and calculated from plasma or urine concentrations. Data are from exposure periods of 24 h or longer.

melting points than hydrocortisone, the penetration of the latter was unaltered (see Table 1). Hydrocortisone has a lower logP of 1.61 and a higher melting point of 219°C, which could potentially be linked to a lower likelihood of precipitating. In addition, the effective absorption is driven by the saturation degree, such that chemicals applied in much lower concentrations than their maximal solubility in a solvent exhibit lower fluxes than their J_{max} values (Williams et al., 2016). Each chemical was tested at the same concentration in PBS and in ethanol. While the saturation degree was similar for the four chemicals in aqueous solution (i.e., between 0.48 and 0.6; reflecting the dose selection of not more than 60% of the maximal aqueous solubility), it was markedly lower in ethanol, in which they were much more soluble. Furthermore, the saturation degree for benzophenone, propylparaben and geraniol were similar (within a factor of 3) and much lower than that for hydrocortisone (i.e., 1.5×10^{-2}). Thus, DD appeared to be affected by using ethanol for chemicals having the lowest saturation degree in this solvent. These results could suggest that the likelihood of increasing the dermal penetration of a chemical by applying it in ethanol is dependent on: (a) rate of solvent evaporation; (b) melting point; (c) logP; and (d) saturation degree in ethanol. This means that of the six chemicals tested only in ethanol (see Table 1), only tetramethyl thiuram disulfide, with a low logP and a high saturation degree would have a DD similar to that when applied in PBS. Potentially, the DD of 2-AAF and 4-BPI may be unaffected by applying them in ethanol if the saturation degree was considered, as these values were similar to that for hydrocortisone (Table 2).

All chemicals were applied as a finite dose, as this is more realistic to the real-life scenario. This means that the majority of the chemicals did not reach a steady-state flux before they were depleted from the donor compartment. These chemicals showed hyperbolic-shaped cumulative RF kinetics profiles, with a plateau reached once the chemical had either been completely absorbed, evaporated or precipitated on the skin surface, or bound to proteins (or a combination of these). Based on the time between application and the point at which the RF kinetics plateaued, it appears that evaporation of the volatile chemicals occurs within about 4 hours. This correlates with the results from volatility tests measuring the fraction of the dose remaining after incubation at room temperature for 4 hours (Grégoire et al., 2019). Additional experiments, in which the evaporation of 10 μL water was measured from the skin surface, indicated that evaporation was complete even after 1 hour (unpublished data). In the studies by Grégoire et al., there was a very good correlation between the percentage recovery of test chemical with the mass balance. The only exception to this correlation was 4-chlorobutyric acid, which appeared to be volatile (50% recovery) in the volatility test but almost the entire applied dose (99%) was recovered in the skin penetration experiment. One reason for this could be the difference in the matrix to which the chemical was added; whereby 4-chlorobutyric acid can evaporate from the surface of a plastic scintillation vial in the volatility test but not from the surface of the skin in the penetration study. Based on the higher amounts of this chemical in the total SC tape strips and epidermis compared with median values, there could be binding, or association, of the chemical with the skin surface that prevents it from evaporating.

The median values for the amount of chemical in the total SC tape strips and epidermis for all 56 chemicals tested was 1.5% and 0.8% of the applied dose, respectively. There were several chemicals that were present in much higher amounts than these, suggesting that they exhibit a reservoir effect in one or both layers. This is not an unusual observation; indeed, there are numerous examples of chemicals that form a reservoir in the skin layers (reviewed by Byford, 2009). The potential for forming a reservoir is reported to be dependent on the extent of protein binding, the rate of penetration through the skin, and the hydro-/lipophilicity of the chemical (Miselnicky, Lichtin, Sakr, & Bronaugh, 1988). In line with this, 4-BPI and triclosan are lipophilic (logP values are 3.48 and 4.76, respectively) and 4-BPI reacts rapidly and extensively with peptides (according to the DPRA) and both chemicals were present in high amounts in the total SC tape strips (17% and 12% of the applied dose, respectively) but in low amounts in the epidermis (1.9% and 2.3% of the applied dose, respectively), suggesting that they form a reservoir in the SC due to their lipophilicity and, in the case of 4-BPI, protein binding, but they do not accumulate in the deeper aqueous layers of the skin. Notably, these chemicals here were also mostly recovered from the skin wash, with DDs of <9% of the applied dose. The accumulation in the SC was not necessarily linked to chemicals with a high lipophilicity, high protein reactivity or slow penetration. For example, IQ is less lipophilic (logP is 1.47), only slowly reacts with peptides in the DPRA (11% depletion of cysteine after 24 hours) and has a lag time of 1.5 hours but was present in high amounts in both the total SC tape strips and epidermis (16% and 8% of the applied dose in the total SC tape strips and epidermis, respectively). Further investigations into reservoir effects are needed, e.g., correlating with partition coefficients, which provides information on the interaction of chemicals with the skin layers (Rothe et al., 2017).

In conclusion, a standard protocol was used to test the penetration of 56 cosmetic-relevant chemicals into and through human skin. While there was some variability due to skin cell outliers and donor variations, the reproducibility of the values was very good. The application of three chemicals in 100% ethanol resulted in a DD that was decreased compared with application in PBS. We attributed this to the more rapid evaporation of the solvent, leading to the precipitation of chemicals with a high logP and a low melting point. RF kinetics reflected the finite dosing and relative volatility of the chemicals, such that a plateau was reached once the chemical had depleted from the donor compartment or had precipitated. While we have made a general analysis of these data, it is hoped that they can be used to: (a) help have a better understanding of factors influencing skin bioavailability of chemicals; (b) help interpret skin toxicity results (sensitization, genotoxicity); and (c) use as input data to test and develop in silico dermal penetration models.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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2-AAF, 2-acetyl aminofluorene; 4-BPI, 4-bromophenyl isocyanate; thiram, tetramethyl thiuram disulphide; PBS, phosphate-buffered saline.

Sources of the physicochemical properties are listed in Table S1 (see Supporting Information). Saturation degree was calculated by dividing the concentration of the dose (in mg/mL) by the maximal solubility in the solvent (mg/mL).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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