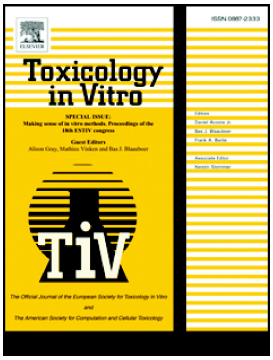

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Dermal absorption of testosterone in human and pig skin *in vitro*

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Abbreviations: ER, Electrical Resistance; LSC, Liquid Scintillation Counting; EFSA, European Food Safety Authority. OECD, Organisation for Economic Co-operation and Development. SCCS, Scientific Committee on Consumer Safety

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1. ABSTRACT

The OECD test guideline 428 for the assessment of dermal absorption *in vitro* has been in force for more than a decade. Various sectors of industry utilise the method for the registration of chemical products. These include the Agrochemical and Cosmetic sectors where the OECD test guideline and industry-specific guidance forms a key part of the human risk assessment process for new and existing products. This investigation has compared the dermal absorption characteristics of one of the OECD 428 reference chemicals, testosterone, in human and pig dermatomed skin. We used identical dosing and skin decontamination conditions for testosterone in Franz static diffusion cells. This included a full mass balance recovery of the dose applied and distribution of the compound in the different layers of the skin. Our investigation has shown that intact human skin provides a more effective barrier to the dermal absorption of testosterone compared with pig skin, when studied according to modern day *in vitro* dermal absorption guidance.

Keywords: Dermal Absorption; Dermatomed skin; Electrical Resistance (ER); OECD 428; Tape stripping; *In vitro* percutaneous absorption

2. INTRODUCTION

The assessment of the dermal absorption potential of active ingredients in cosmetic, agrochemical and industrial chemical products that come into contact with the skin is a key part of human risk assessment. This ensures that under normal, or expected conditions, based on the hazard potential of a particular chemical, there is a sufficient margin of safety for manufacturers, handlers and end users of products who may be exposed to products containing the active chemical. The assessment of dermal absorption for human risk assessment follow the OECD 428 test guideline which almost exclusively involves *in vitro*

techniques that utilises donated human or animal skin. There are now very few dermal absorption studies undertaken *in vivo,* since *in vitro* studies using resected skin have been shown to be predictive of man for a variety of chemicals across multiple industrial sectors (WHO, 2006). The OECD 428 approach is now used extensively, particularly by the personal care/cosmetic and agrochemical industries, and forms a key part of the human risk assessment and registration process for new and existing chemicals that come into contact with the skin either intentionally or during their occupational use.

The OECD 428 test guideline has been used for many years as a stand-alone *in vitro* method for predicting dermal absorption (OECD, 2004a) and this has led to a major reduction in animal use in this area of toxicology and new product development. Current regulations for the registration of consumer products and agrochemicals in the European Union use industry-specific protocols for dermal penetration that are aligned to the methods described in OECD 428. For example, the Scientific Committee on Consumer Safety for cosmetic ingredients (SCCS, 2010) and European Food Safety Authority for pesticide-containing products (EFSA, 2012) have their own additional dermal absorption guidance for cosmetic and agrochemical products, respectively.

Each of these industry-specific guidance documents permits the use of alternative species to human skin. In the case of agrochemicals, rat skin is sometimes used, not because it is recognised as a good model for man, far from it, but in order to bridge between historical or contemporary data that has been generated on the product in the rat *in vivo* (EFSA, 2012). In the case of personal care and cosmetic products where animal studies are not used, the SCCS guidance (SCCS, 2010) also permits the use of pig skin *in vitro*, in addition, to the more conventional human skin models. The pig is regarded as being a reasonable surrogate for human skin with regard to skin permeability characteristics and morphology (Benech-Kieffer *et al*, 2000; Gerstel *et al*, 2016; Dick and

Scott, 1992; WHO, 2006). Pig skin is also used for formulation development and product selection by the pharmaceutical industry ahead of the more costly human studies (Herkenne *et al*, 2016). It was therefore of interest to compare human and pig skin side-by-side in an identical OECD 428 protocol, based largely on the EFSA Guidance recommendations, which include an *interim* decontamination step and full mass balance recovery of the chemical in question, 24 hours following skin exposure.

Testosterone was selected as the representative chemical for this comparative study since it is one of the reference chemicals recommended in the OECD test guideline 428 to be tested periodically to demonstrate the performance of the method, particularly in laboratories that are less familiar with the *in vitro* method (OECD, 2004a). Furthermore testosterone has been widely studied over many years in human *in vitro* and *in vivo* models (Bronaugh and Franz, 1986) and as a therapeutic agent for a variety of medical purposes (Hadgraft and Lane, 2015). Although, our own laboratory (Heylings *et al*., 2007) and others (van de Sandt *et al*, 2004) have compared the dermal penetration properties of testosterone and other standard reference chemicals in different species in previous multi-laboratory investigations, a new up-to-date evaluation of the OECD 428 approach using dermatomed skin and other methodological aspects that have been introduced over the years, may prove useful for others involved with these types of studies.

Regulatory studies of *in vitro* dermal absorption undertaken in the 1990s and early 2000s often involved the use of heat-separated epidermal membranes (ECETOC 1993, ECVAM 1996). However, industry guidance now recommends the use of split-thickness skin, which is prepared with a dermatome (EFSA 2012; SCCS, 2010). This produces skin preparations that have not only the *stratum corneum* barrier and underlying epidermis, but a thin layer of dermis beneath that is in contact with the receptor fluid. In these modern day studies, the “absorbed dose”, or that which is regarded as systemically available, includes

the test chemical remaining in the skin, following tape stripping, and is not assessed by flux alone, which only measures the test chemical in the receptor fluid over a time course. Consequently, dermatomed skin that forms the basis for this investigation has become the main skin preparation technique for regulatory studies and use of a finite (low volume) application of the product containing the test chemical (Heylings, 2015).

3. METHODS

3.1 Preparation of dermatomed skin membranes

Human skin used for this investigation was approved by the NHS Local Research Ethics Committee and donated tissue was supplied by The National Disease Research Interchange (NDRI), Philadelphia, USA. We use a strict protocol for the sourcing of human tissue. We utilise the abdominal region for these studies since the skin permeability, thickness of the stratum corneum as well as follicle density and depth have intermediate properties, compared with other regions of the human body. In addition it is important that the skin is not swabbed with any chemicals such as iodine or ethanol prior to freezing. Since an important aspect of any human investigation is the inter-subject response, skin samples from at least 4 different human donors were used. Care was taken not to use any area of the skin that appeared abnormal or where the sample had been folded prior to freezing. Pig skin preparations used for this investigation were from animals of the British White strain of pig (aged 6-8 weeks) that had been bred for food and were sourced from a local abattoir. The flank region of the animal was used in these investigations. As part of the experimental design we ensured that at least 4 different animals were used in the dose group. Skin membranes (approximately 6 cm diameter) from both species were cut at a thickness of 400 µm using an electric dermatome. The edges of the skin sample were avoided to ensure uniform thickness of each specimen. Each skin membrane was given a

unique identifying number and stored frozen, at -20^{o}C, on aluminium foil, until required for use.

3.2 In vitro static diffusion cell equipment and measurement of skin integrity

Details of the diffusion cell assembly used in these investigations are described in the OECD Guideline 428 (OECD, 2004a) and Guidance Document No. 28 (OECD, 2004b). The test guideline shows a diagram of our modified glass static diffusion cell, based on the original design of this device (Franz, 1975). Other publications from our laboratory describe the type of glass static diffusion cells in more detail (Scott and Clowes, 1992), and the mass balance procedure used to define systemic exposure in modern day studies (Heylings, 2014). Our automated static diffusion cell system is depicted pictorially in more recent publications (Heylings, 2015a).

Discs of dermatomed human or pig skin approximately 3.3 cm diameter were mounted dermal side down. Each cell had an exposed area of skin of 2.54 cm^2. The receptor chambers were filled with a recorded volume of physiological saline, containing 5% bovine serum albumin and 0.1% sodium azide. The preparations were placed on a magnetic stirrer plate and semi-immersed in a water bath maintained at a skin temperature of 32 ± 1^{o}C. Prior to use, the skin integrity of each membrane was assessed to ensure that each skin sample had not been damaged during procurement, shipping, storage or during the dermatoming procedure. It should be stressed that the assessment of skin integrity is probably the most important aspect of the whole *in vitro* dermal absorption method. Not only is it an OECD guideline requirement, but laboratories need to ensure that they are applying test compounds to skin with an intact barrier. In this investigation, skin integrity assessment was undertaken by measuring the Electrical Resistance (ER) of each sample in the diffusion cell and the acceptance/rejection criteria established previously for

normal intact skin in our laboratory for different species (Heylings *et al*., 2001; Davies *et al*., 2004) in our static diffusion cells. Resistance across each membrane was measured using a PRISM Electronics AIM6401 LCR data bridge and was expressed as kΩ/cell area. This method measures the resistance (or impedance) of skin samples in diffusion chambers and has been shown by several laboratories to be representative of skin barrier function (Lawrence *et al*., 1997; Davies *et al*., 2004; WHO, 2006; Heylings, 2012). The glass diffusion chambers containing human or pig skin were allowed to equilibrate in a water bath at 32^{o}C for approximately 30 minutes. Following this, one electrode was inserted into 4.5ml of saline via the receptor chamber side arm and another electrode was placed into 2ml of saline pipetted into the donor chamber. Once stabilised, the resistance value for each skin sample was recorded. The saline in the donor chamber was then removed and the skin allowed to dry naturally. Previous studies in our laboratory had established the ER values for intact skin from different species and different methods of skin preparation. These cut-off values for intact skin were established in our 2.54cm^2 surface area Franz static diffusion cells as used in this investigation (Davies *et al*., 2004). Any human skin membranes that had an ER value below 10 kΩ or below 3 kΩ for pig skin were regarded as having poor skin integrity for that particular species and were not used in these investigations.

3.3 Application of Testosterone

Unlabelled testosterone was obtained from Sigma-Aldrich (The Old Brickyard, New Road Gillingham, Dorset. UK) and [4-^{14}C]-testosterone was obtained from Quotient Bioresearch (Radiochemicals) Ltd, Cardiff, UK. The receptor chambers of the cells were filled with a recorded volume of 0.9% aqueous NaCl + 5% bovine serum albumin + 0.01% sodium azide. A mixture of unlabelled and [^{14}C]-testosterone was dissolved in 40% ethanol

in water to make a 1mg/ml dosing solution with a radioactivity content of approximately 2 MBq/mL. The purity and testosterone concentration of the dose preparations (40% ethanol : 60% water w/w) was confirmed by HPLC and radiochemical detection by LSC. A finite skin application rate of 10 µL/cm^2 was used, equivalent to a dose of 10 µg testosterone/cm^2. This was applied to the surface of the skin membranes. In the case of human skin we dosed four pairs human donor skin in order to be able to investigate any inter-subject differences in response. The glass diffusion cells were placed semi-immersed in a water bath maintained at 32^{o}C ± 1^{o}C. The receptor fluid beneath the skin was constantly mixed with a magnetic stirrer so the auto-sampler collection of a small volume could be used to calculate the amount of testosterone that had penetrated into the receptor fluid at any given time point.

Samples (0.5 mL) of the receptor fluid were taken manually immediately before dosing and then at 1, 2, 3, 4, 5, 6, 7 and 8 h, using an auto-sampler. The volume of fluid in the receptor chamber was maintained by the replacement of a volume of receptor fluid, equal to the sample volume immediately after each sample was taken. At the scheduled *interim* washing time of 8 h, a mild skin washing procedure was performed which involved the swabbing of the skin surface firstly with sponges pre-wetted with a dilute soap solution and then sponges pre-wetted with water. Finally, the skin surface was rinsed with a single aliquot of water before returning the cells to the water-bath. Further samples of receptor fluid were collected at 10, 12, 14, 16, 18, 20, 22 and 24 h after application and analysed for testosterone. At 24 h, the donor chamber was carefully detached from the cell assembly and the terminal skin washing procedure and mass balance was performed. The skin washing regime involved the same sponge and water wash described previously after which the skin was allowed to dry naturally prior to removal of the *stratum corneum* by tape stripping. The skin stripping method followed the standard approach described in test

guideline OECD 428 (Trebilcock *et al*., 1994), using repeated application of adhesive tape (Scotch 3M Magic Tape, 1.9 cm wide). A maximum of 15 individual tape strips were taken from each skin sample and the testosterone content in each strip was measured by LSC, following extraction from the tape.

The flange skin (the unexposed/undosed skin which had been held between the donor and receptor chamber) was cut away and the epidermis was separated from the dermis by heat separation. Each sample of epidermis and dermis was separately solubilised with tissue digestant prior to analysis for testosterone by LSC, following the addition of scintillation fluid. Likewise, the radioactivity content of each of the receptor fluid samples was also determined at each time point.

3.4 Calculations and statistical analyses

The time-course absorption of testosterone into the receptor fluid was calculated by dividing the amount of test penetrant at each time point by the area of exposed dermatomed skin (2.54 cm^2) and plotting the results as amount of testosterone absorbed ($\mu g/cm^2$) versus time (h). The slope of the absorption profile between given time points provided the rate of absorption of testosterone per cm^2 of the skin ($\mu g/cm^2/h$) during that period. The proportion ($\mu g/cm^2$) of testosterone that was recovered from the individual compartments of the system (donor chamber, skin wash, tape strips, flange, epidermis, dermis and receptor fluid) was expressed as a percentage of the actual amount applied to the surface of each skin membrane. This allowed a full mass balance distribution of testosterone to be determined in accordance with the OECD 428 test guideline. The human and pig skin values were expressed as mean ± SD for each compartment. A comparison was made between the dermal absorption observed in each species using Student's *t*-test for unpaired variates.

4. RESULTS

4.1 Skin Penetration of Testosterone through Human Dermatomed Skin

The dermal absorption of testosterone through human skin into the receptor fluid gave a typical exponential type time course profile (Figure 1). After a lag phase of about 1 hour there was a relatively fast phase of absorption. The *interim* wash at 8h, which is part of the EFSA 2017 guidance for pesticide-containing products had no effect on general shape of the curve. The mean maximum absorption rate of testosterone was 0.027 µg/cm^2/h, n=8, which occurred during the first 12 hours. This was followed by a slower phase of testosterone absorption from 12-24 hours, with a mean rate of 0.005 µg/cm^2/h. This time course profile is typical of finite dose applications where the solvent (or vehicle) used is gradually lost from the skin surface. This, in turn, can reduce the ability of the substance to move into and through the *stratum corneum*, as the dose dries onto the skin surface. The absorption profile was similar across the 4 different human donors used and accounted for 2.45% of the applied dose over 24h. This was equivalent to a mean value of 0.246 µg testosterone/cm^2, when expressed as mass per skin area for the 10 µg/cm^2 dose application in the 8 replicates.

OECD test guideline 428 also requires a full mass balance distribution of the applied material at 24 h. This includes the proportion of the dose that is left in the remaining epidermis and dermis following tape stripping and removal of the *stratum corneum*. This proportion of the dose applied (mean of 0.73%) is regarded as being potentially systemically available in a regulatory study on pesticide-containing products (EFSA, 2017). In the human skin part of this investigation, the mean total systemically available dose was 3.17% of that applied to the skin. The vast majority (94.2%) of the dose was washed off the skin at 8 h with a further 1.34% removed from the surface in the terminal skin wash at

24 h. The 15 tape strips of the *stratum corneum* amounted to a further 0.16% of the dose with 0.73% found in the remaining skin, comprising the epidermis and dermis. A mean total recovery of 99.0 ± 0.65% (mean ± SD) was achieved with the 8 replicates. The mass balance recovery of testosterone in each compartment of human skin is shown in Table 1. In order to see the distribution across the human skin samples, the individual data for each skin diffusion cell and each compartment of the investigation is shown in Table 2.

4.2 Skin Penetration of Testosterone through Pig Dermatomed Skin

The time course profile of dermal absorption of testosterone through pig skin is shown in Figure 2. Interestingly, the skin penetration profile in pig skin was almost identical to that observed in human skin in terms of lag phase and shape of the curve. As with human skin, the 8h *interim* skin surface wash had no effect on the general shape of the absorption curve. The clear difference from the human skin investigation was the much greater proportion of the applied dose of testosterone that was found in the receptor fluid in pig skin at 24 h. In fact, the absorption of testosterone through pig skin was between 5 and 6 times higher than through human skin. A total of 13.1% of the dose, equivalent to 1.33 µg testosterone/cm^2, had reached the receptor fluid by 24 h. The majority of the dose that was found in the receptor fluid had penetrated the skin samples by 12 h, with an average flux over 24h of 0.055 µg/cm^2/h, n=11. The time course showed the typical "saturation" type profile of dermal absorption with finite (low volume) doses.

The systemically available dose that includes the dose that has penetrated to the epidermis and dermis, in addition to the receptor fluid was 15.8% of the dose applied to the skin. As with human skin, the majority of the applied dose (76.6%) could be washed off the skin at 8 h and a further 5.1% was removed in the terminal wash at 24 h. The 15 tape strips of *stratum corneum* amounted to a further 0.23% of the dose. Only 2.63% of the

testosterone dose was found in the remaining skin after tape stripping. A mean total recovery of 98 ± 1.6% (mean ± SD) was achieved for the 11 pig skin replicates. The mass balance distribution and recovery of testosterone in pig skin is shown in Table 1. The individual pig skin data for skin diffusion cell and each compartment of the investigation is shown in Table 3.

5. DISCUSSION

This investigation has compared the dermal penetration and skin distribution of an identical dose application of the OECD 428 reference chemical, testosterone in both human and pig skin. Our experiments followed the experimental design described in the *in vitro* test guideline, OECD 428, using *ex vivo* tissue from multiple human donors or animals, in the case of the pig. The actual protocol used here included an *interim* decontamination of the skin surface at 8 h, as required for the testing of crop protection products (EFSA, 2017). This intermediate washing step is also part of the SCCS guidance for dermal absorption for the evaluation of specific cosmetic products that have short exposure times such as hair dyes where an *interim* skin washing step is normally undertaken 30 min following skin application (SCCS, 2010). For the various industrial sectors that utilise guideline OECD 428 the key aspect of the study protocol is to mimic real or expected exposure to the product containing the active ingredient. For pesticides this is the normal working day, where either the product concentrate or spray strength dilutions are handled by the Operator, over, for example, an 8h period. In contrast, most personal care products such as skin creams are “leave-on” and the duration of exposure is for the full 24h, with no *interim* skin washing step in the study design. In order to make this particular investigation more comprehensive we tape stripped the *stratum corneum* 15 times rather the conventional 5 strips used in the EFSA guidance for dermal absorption.

This allowed is to profile testosterone more accurately across this outer layer of the skin in the two species studied.

The primary observation of this investigation was the difference in the extent of dermal absorption between the two species, with human skin offering a more effective barrier preventing skin penetration of testosterone into the receptor fluid when compared with pig skin under identical experimental conditions. The magnitude of difference in dermal absorption was between 5 and 6-fold for this particular compound. Interestingly, despite the difference in the extent of absorption between species, the time course profile of absorption into the receptor fluid was very similar. The mass balance distribution provides a good explanation for the species difference in testosterone absorption. The first step in the dermal absorption process is the ability of the test penetrant, in its vehicle, to penetrate into and traverse the outer layers of the *stratum corneum*. Indeed, the vehicle itself can have a major impact on the overall skin penetration process since the first step involves partitioning out of the application dose and into the lipid rich outer layers of the *stratum corneum*. This vehicle effect has been shown for a number of compounds of differing physicochemical properties in human skin and in human volunteer studies (Trebilcock *et al*, 1994). In the case of testosterone here, the *interim* surface decontamination with soap and water at 8 h is likely to prevent the applied dose remaining on the surface from gaining further access to the skin after this time. In addition, the soap washing procedure and mechanical action of the sponges used to swab the surface, probably also removes a significant proportion of any testosterone in the micro-folds, appendages and, to a certain extent, any unabsorbed compound that is bound to the lipid-rich areas of desquamed *stratum corneum*.

The difference in the quantity of testosterone removed by the soap wash at 8 h was quite marked and significantly different ($p<0.01$) between the two species. A total of 94% of the

applied testosterone dose could be removed from human skin, but only 77% could be removed from pig skin using an identical procedure undertaken by the same Technician at 8 h. This more effective penetration into the surface of pig skin in the first few hours following exposure leads to higher proportions of the testosterone dose reaching all the layers beyond the *stratum corneum* with the underlying epidermis, dermis and receptor fluid giving considerably higher values in pig skin compared with human skin. Consequently, the systemic exposure for testosterone, which, using the EFSA guidance description, which includes the proportion of the dose in the regions beyond the tape stripped *stratum corneum*, as “potentially absorbable” was significantly higher in pig skin at 15.8 ± 5.91%, compared with human skin 3.17 ± 2.40% ($p < 0.01$).

A key parameter in these dermal absorption and skin distribution investigations, and a requirement by the OECD 428 guideline, and associated industry-specific guidance, is an acceptable mass balance recovery of the applied dose. This “validates” the experiment by accounting for all the test material that was applied to the skin. In this investigation, a very good mass balance recovery of testosterone was achieved in both species with 99.0 ± 0.65% recovered in human skin and 98.0 ± 1.58% recovered in pig skin (values are mean ± SD, Table 1). This is one of the important study performance criteria in the OECD 428 test guideline, where a mean total recovery of 95-105% of the active ingredient applied is required when the study forms part of a regulatory submission for a new or existing product as part of the human risk assessment. When there is a shortfall in total recovery of the applied dose, the regulatory authorities are obliged to presume that any missing dose may have been in one of the systemically available compartments. Therefore, in the spirit of conservatism, the missing fraction of the dose applied is added to the absorbed dose in the absence of other information. This ensures that in a human risk assessment that systemic exposure is not underestimated. In the case of volatile actives

an unoccluding carbon filter trap above the diffusion cells is sometimes used to ensure a good mass balance recovery. This provides the evidence on the proportion of the dose that was clearly unabsorbed. For drug absorption and dermal delivery investigations that utilise a similar *in vitro* protocol aligned to OECD 428, this conservative approach of including the "missing" dose in a mass balance as being systemically available is not appropriate since the objective is often to maximise the dermal absorption of the active from the formulated product.

The design of the present investigation followed the guidance on the numbers of individual human donor skins used, and indeed, the number of different animals used when these studies are used for regulatory submissions to various authorities such as EFSA or SCCS. There is a requirement for human skin absorption studies that tissue should be procured from a minimum of four different human donors for each dose of an active ingredient. This distribution of donor and animal skin was followed in this investigation and ensured that any intrinsic human donor effect on dermal absorption is incorporated into the experimental design. In this investigation one of the human donors showed a slightly different skin distribution of testosterone to the other human samples in the group. If you examine Table 3, the diffusion cells 84 and 85 contained skin from the same human donor (Donor 4). Using the same washing procedure across the group, a similar but lower amount was removed from that donor's skin at 8h. This only amounted to a few percent of the dose applied. However, the consequences were a higher amount of testosterone found in the skin at 24h and also higher amounts of testosterone was present in the receptor fluid for that particular donor.

The overall mass balance for these two replicates was very good and close to 100%, which indicates that this was not a technical or analytical issue and much more likely to be indicative of different skin permeability properties, at least to testosterone, across the

population. This endorses the need to include several human donors for each dose level of a compound being tested using the OECD 428 *in vitro* dermal absorption protocol. This issue of variability in data from *in vitro* dermal absorption studies both for the distribution across the compartments of the study and the overall systemic exposure values across replicates is inherent to this study type and not often recognised when various statistical methods are used with such data. In the case of animal skin studies of dermal absorption it is usual for the same genetic strain, age, sex and supplier of the animal skin samples to be used. Therefore, dermal absorption studies with animal skin are less likely to provide the population variability that we observe regularly with skin from different human donors, and indeed from different sites of the human body (WHO, 2006). The key aspect here, and this applies to all species, is the importance of ensuring that the skin is intact and not damaged by the collection, storage and dermatoming of the specimens (Heylings, 2012; Davies *et al*, 2015). This can have a much greater effect on the overall dermal absorption value obtained using the OECD 428 approach.

It should be noted that the species difference in dermal absorption observed here relates to a single dose of one test chemical, testosterone. Furthermore, it was applied to the skin in an aqueous ethanol vehicle, in order to ensure that the reasonably lipophilic chemical was dissolved completely and homogenous in the 10µl/cm^2 finite dose. It is therefore possible that use of a different vehicle and indeed, chemicals with different lipophilicities that the species difference observed here between human and pig skin may not be as marked under different conditions and with different chemicals. However, it is generally accepted that human skin has the most effective barrier to skin penetration across mammalian species for chemicals in general, whether they be polar or lipophilic (OECD 2004a; OECD 2004b; WHO, 2006). However, in this particular investigation we have shown that intact human skin is a more effective barrier to the dermal absorption of

testosterone compared with pig skin when studied side by side and according to modern day *in vitro* dermal absorption guidance.

Prior to the publication of the OECD test guidelines for dermal absorption in 2004, skin integrity investigations comparing the permeability properties of different species had already identified that each species had different skin permeability properties. The skin barrier properties to tritiated water, trans-epidermal water loss and electrical resistance were somewhat lower in pig skin, but certainly closer to human skin than rat skin (Davies *et al*, 2004; Heylings, 2012). Pig skin is used as surrogate for human skin for regulatory *in vitro* dermal absorption studies on personal care products (Benech-Kieffer, *et al*, 2000; Gerstel *et al*. 2016; SCCS, 2010). Porcine skin is also a useful model for the screening of different formulations containing the same active, where the objective of the investigation is to identify adjuvants that provide the best profile of absorption and distribution alongside other end points that may be a diverse as efficacy in crop protection or efficacy relating to drug delivery. In the case of drug research, a well-controlled *in vitro* pig skin protocol, according to the principles of OECD 428l is often used as a screen to select and optimise formulations ahead of a more costly human volunteer study (Herkenne *et al*. 2007). Ensuring that the skin permeability is normal in these *in vitro* skin studies is just as important in the development of topical pharmaceuticals as it is for safety studies with industrial chemicals. Therefore, the OECD 428 *in vitro* dermal absorption method, when conducted correctly, has a wide range of applications in both human risk and human benefit.

6. REFERENCES

Benech-Kieffer, F., Wegrich, P., Schwarzenbach, R., Klecak, G., Weber, T., and Schaefer, H., 2000. Percutaneous absorption of sunscreens *in vitro*: Interspecies comparison, skin models and reproducibility aspects. Skin Pharmacol. Appl. Skin Physiol. 13, 324-335.

Bronaugh, R. L. and Franz, T.J., 1986. Vehicle effects on percutaneous absorption: *in vivo* and *in vitro* comparisons with human skin. Br. J. Dermatol. 115, 1-11.

Davies, D.J., Ward, R.J., Heylings, J.R., 2004. Multi-species assessment of electrical resistance as a skin integrity marker for *in vitro* percutaneous absorption studies. Toxicol. In Vitro 18, 351-358.

Davies, D.J., Heylings, J.R., McCarthy, T.J., Correa, C.M., 2015. Development of an *in vitro* model for studying the penetration of chemicals through compromised skin. Toxicol. In Vitro 29, 176-181.

Davies, D. J., Heylings, J. R., Gayes, H., McCarthy, T. J. and Mack, M. C., 2017. Further development of an *in vitro* model for studying the penetration of chemicals through compromised skin. Toxicol. In Vitro, 38, 101-107.

Dick, I.P., Scott, R.C., 1992. Pig ear as an *in vitro* model for human skin permeability. J. Pharm. Pharmacol. 44, 640-645.

ECETOC, 1993. European Centre for Ecotoxicology and Toxicology of Chemicals. Monograph No. 20, Percutaneous Absorption, August 1993.

ECVAM, 1996. European Committee for the Validation of Alternative Methods. Workshop Report and Recommendations on Methods for Assessing Percutaneous Absorption. ATLA 24, pp. 81-106.

EFSA, 2017. Buist H, Craig P, Dewhurst I, Hougaard Bennekou S, Kneuer C, Machera K, Pieper C, Court Marques D, Guillot G, Ruffo F and Chiusolo A, 2017. Guidance on Dermal Absorption. EFSA Journal 15(6):4873, 60 pp.

Franz, T.J., 1975. Percutaneous absorption on the relevance of *in vitro* data. J. Invest. Dermatol. 64, 190-195.

Gerstel, D., Jacques-Jamin, C., Schepky, A., Cubberley, R., Eilstein, J., Gregoire, S., Hewitt, N., Klaric, M., Rothe, H., and Duplan, H., 2016. Comparison of protocols for measuring cosmetic ingredient distribution in human and pig skin. Toxicol. In Vitro 34, 153-160.

Hadgraft, J. and Lane, M.E., 2015. Transdermal delivery of testosterone. Eur. J. Pharm. Biopharm. 92, 42-48.

Herkenne, C., Naik, A., Kalia, Y.N., Hadgraft, J., and Guy, R.H., 2007. Ibuprofen Transport into and through Skin from Topical Formulations: *In Vitro-In Vivo* Comparison. J Invest. Dermatol. 127, 135-142.

Heylings, J.R., Ward, R.J. and Hughes, L., 2001. Comparison of tissue sources for the skin integrity function test (SIFT). Toxicol. In Vitro 15, 597-600.

Heylings, J. R. and Esdaile, D. J., 2007. Dermal Absorption of Pesticides. In: Dermal Absorption and Risk Assessment. 2nd Ed. Edited by: Roberts, M. S. and Walters, K. A. Decker.

Heylings, J. R., 2012. Risk Assessment. In Topical and Transdermal Drug Delivery: Principles and Practice. Edited by: Benson, H. A.E., and Watkinson, A. C., Wiley & Sons Inc. pp183-199.

Heylings, J. R., 2014. Diffusion Cell Design. In: Topical Drug Bioavailability, Bioequivalence, and Penetration. 2nd Ed. Edited by Shah, V. P., Maibach, H. I. and Jenner, J. Springer Science and Business Media, New York.

Heylings, J. R., 2015a. Dermal Penetration: A Regulatory Testing Overview. Published in the on-line journal AltTtox. http://alttox.org/mapp/toxicity-endpoints-tests/dermal-penetration/

Heylings, J.R., 2015b. Dermal Penetration: Emerging Science and Policy. Published in the on-line journal AltTtox. http://alttox.org/mapp/toxicity-endpoints- tests/dermal-penetration/emerging-science-policy/

Lawrence, J.N., 1997. Electrical resistance and tritiated water permeability as indicators of barrier integrity of *in vitro* human skin. Toxicology In Vitro, 11, 241-249.

OECD, 2004a. Test Guideline 428. Skin Absorption: *In Vitro* Method. Organisation for Economic Co-operation and Development, Paris.

OECD, 2004b. Guidance Document No. 28. The Conduct of Skin Absorption Studies. Organisation for Economic Co-operation and Development, Paris.

van de Sandt, J.,J., M., van Burgsteden, J.A., Cage, S., Carmichael, P., L., Dick, I., Kenyon, G., Korinth, G., Larese, F., Limasset, J.C., Maas, W. J. M., Montomoli, L., Nielsen, J.B. and Payan, J-P., 2004. *In vitro* predictions of skin absorption of caffeine, testosterone, and benzoic acid: a multi-centre comparison study. Regulatory Toxicology and Pharmacology 39, 271-281.

SCCS, 2010. Scientific Committee on Consumer Safety. Opinion on Basic Criteria for the *In Vitro* Assessment of Dermal Absorption of Cosmetic Ingredients. Updated 22nd June 2010. SCCS/1358/10.

Scott, R.C., Clowes, H.M., 1992. *In vitro* percutaneous experiments: A guide to the technique for use in toxicology assessments. Toxicol. Methods 2, 113-123.

Trebilcock, K.L., Heylings, J.R., Wilks, M.F., 1994. *In vitro* tape stripping as a model for *in vivo* skin stripping. Toxicol. In Vitro 8, (4), 665-667.

WHO (World Health Organisation), 2006. Dermal Absorption. IPCS Environmental Health Criteria 235. ISBN 92-4-157235. WHO, Geneva.

Table 1: Mass Balance Distribution of Testosterone in Human and Pig Skin

Test Compartment	Human skin		Pig skin	
	%	SD	%	SD
Skin wash at 8 hours	94.2	3.15	76.6	10.4
Skin wash at 24 hours	1.34	1.26	5.13	4.45
Stratum corneum	0.16	0.07	0.23	0.17
Remaining epidermis	0.41	0.43	0.89	0.68
Dermis	0.32	0.30	1.74	1.22
Receptor fluid	2.45	2.05	13.1	4.99
Systemically available	3.17	2.40	15.8	5.91
Total recovered	99.0	0.65	98.0	1.58

Mean % dose applied ± Standard Deviation (SD) for each compartment
Testosterone dose applied to skin = 1.0 mg/ml in ethanol/water vehicle
Interim skin wash at 8 hours; Mass balance recovery at 24 hours
Human skin n=8 replicates (4 donors); Pig skin n=11 replicates (6 animals)
Systemically available = Receptor fluid + Remaining epidermis + Dermis

Table 2. Human Skin: Testosterone (1mg/mL)

Test Compartment	Percent of Dose Recovered (%)									
	Cell 31 Donor 1	Cell 43 Donor 1	Cell 47 Donor 2	Cell 48 Donor 2	Cell 53 Donor 3	Cell 54 Donor 3	Cell 84 Donor 4	Cell 85 Donor 4	Mean	SD
8h Wash Pipette	<LOD	0.017¤	0.005¤	0.007¤	0.030	<LOD	0.230	0.008¤	0.049	0.089
Skin Wash @ 8h	97.3	96.9	93.0	97.4	95.6	93.9	89.8	89.5	94.2	3.19
Total 8h Wash	97.3	96.9	93.0	97.4	95.6	93.9	90.0	89.5	94.2	3.15
Donor Chamber	0.034	0.104	0.039	0.020¤	0.012¤	0.005¤	0.225	0.216	0.082	0.091
Skin Wash @ 24h	0.437	0.924	2.17	0.556	0.647	0.360	4.05	1.58	1.34	1.26
Tape Strips										
1	0.023	0.015	0.020	0.010¤	0.031	0.005¤	0.055	0.014	0.022	0.016
2	0.017	0.011¤	0.024	0.011¤	0.027	0.005¤	0.028	<LOD	0.018	0.009
3	0.006¤	0.010¤	0.013	0.014	0.014	0.003¤	0.015	<LOD	0.011¤	0.004
4	0.014	0.014	0.024	0.013	0.015	0.004¤	0.024	0.017	0.016	0.006
5	0.010¤	0.011¤	0.016	0.010¤	0.012	0.008¤	0.012	0.007¤	0.011¤	0.003
6	0.008¤	0.005¤	0.012	0.011¤	0.010¤	0.003¤	0.017	0.007¤	0.009¤	0.004
7	0.013	0.012	0.016	0.012	0.007¤	0.005¤	0.012	0.006¤	0.010¤	0.004
8	0.013	0.009¤	0.010¤	0.007¤	0.008¤	0.001¤	0.012	0.009¤	0.009¤	0.004
9	0.008¤	0.007¤	0.013	0.007¤	0.002¤	0.001¤	0.063	0.011¤	0.014	0.020
10	0.008¤	0.006¤	0.017	0.004¤	0.006¤	0.002¤	0.009¤	0.005¤	0.007¤	0.004
11	0.004¤	0.008¤	0.012	0.002¤	0.003¤	0.007¤	0.017	0.005¤	0.007¤	0.005
12	0.001	0.005¤	0.008¤	0.003¤	0.002¤	0.0001¤	0.014	0.010¤	0.005¤	0.005
13	0.003¤	0.008¤	0.017	0.007¤	0.004¤	0.007¤	0.007¤	0.023	0.010¤	0.007
14	0.005¤	0.005¤	0.020	0.007¤	0.013	0.003¤	0.008¤	0.004¤	0.008¤	0.006
15	0.009¤	0.015	0.005¤	0.009¤	0.003¤	0.005¤	0.007¤	0.001¤	0.007¤	0.004
Stratum Corneum (Total Tape Strips)	0.142	0.144	0.228	0.126	0.156	0.060	0.300	0.116	0.159	0.074
Flange	0.004¤	0.024	0.099	0.014	0.029	0.017	0.202	0.109	0.062	0.069
Dermis	0.062	0.142	0.264	0.056	0.133	0.155	0.496	0.738	0.256	0.241
Skin Membrane^	0.065	0.166	0.363	0.070	0.162	0.172	0.698	0.847	0.318	0.298
Remaining Epidermis	0.024	0.435	1.38	0.209	0.176	0.116	0.465	0.440	0.405	0.426
Receptor Fluid	0.585	0.735	1.75	0.545	2.47	3.06	4.03	6.44	2.45	2.05
Systemically available*	0.674	1.34	3.49	0.824	2.81	3.34	5.19	7.72	3.17	2.40
TOTAL	98.6	99.4	99.0	98.9	99.2	97.6	99.8	99.2	99.0	0.645

^Skin Membrane = Sum of Dermis and Flange (Unexposed skin)

*Systemically available = Sum of Skin Membrane, Receptor Fluid and Remaining Epidermis (values below the double line)

¤ = Less than LOQ (Limit of Quantification)

Table 3. Pig Skin: Testosterone (1mg/mL)

Test Compartment	Percent of Dose Recovered (%)												
	Cell 43 Animal 1	Cell 47 Animal 2	Cell 48 Animal 2	Cell 55 Animal 3	Cell 62 Animal 4	Cell 64 Animal 4	Cell 67 Animal 4	Cell 71 Animal 5	Cell 85 Animal 1	Cell 86 Animal 1	Cell 98 Animal 6	Mean	SD
8h Wash Pipette	0.017	0.215	0.020	0.020	0.051	0.036	0.088	0.013	0.015	0.009¤	0.173	0.060	0.071
Skin Wash @ 8h	83.2	55.4	85.5	64.2	67.6	78.8	77.7	82.4	85.3	88.8	73.3	76.6	10.4
Total 8h Wash	83.2	55.7	85.6	64.3	67.6	78.8	77.8	82.4	85.3	88.8	73.5	76.6	10.3
Donor Chamber	0.111	0.265	0.031	0.539	1.05	0.099	0.248	0.056	0.236	0.045	0.255	0.267	0.299
Skin Wash @ 24h	1.55	11.3	0.944	8.14	12.1	1.89	9.32	1.67	2.41	0.422	6.70	5.13	4.45
Tape Strips													
1	0.021	0.017	0.040	0.006¤	0.009¤	0.004¤	0.066	0.005¤	0.008¤	0.012¤	0.011¤	0.018	0.019
2	0.013	0.016	0.050	0.019	0.019	0.006¤	0.126	0.005¤	0.014	0.010¤	0.008¤	0.026	0.035
3	0.012	0.041	0.022	0.027	0.038	0.005¤	0.022	0.001¤	0.004¤	0.005¤	0.005¤	0.017	0.014
4	0.027	0.036	0.020	0.009¤	0.025	0.006¤	0.019	0.006¤	0.008¤	0.007¤	0.006¤	0.015	0.011
5	0.019	0.033	0.014	0.016	0.030	0.003¤	0.024	0.026	0.012¤	0.006¤	0.005¤	0.017	0.010
6	0.008¤	0.066	0.026	0.016	0.022	0.004¤	0.033	0.003¤	0.003¤	0.003¤	0.047	0.021	0.021
7	0.004¤	0.009¤	0.012	0.012	0.035	0.003¤	0.044	0.0009¤	0.002¤	0.004¤	0.018	0.013	0.014
8	0.006¤	0.021	0.011¤	0.010¤	0.021	0.005¤	0.022	0.003¤	0.003¤	0.003¤	0.008¤	0.010¤	0.008
9	0.009¤	0.014	0.013	0.016	0.066	0.0005¤	0.030	0.001¤	0.003¤	0.003¤	0.040	0.018	0.020
10	0.012	0.040	0.010¤	0.012	0.034	0.004¤	0.019	0.004¤	0.0009¤	0.004¤	0.032	0.016	0.014
11	0.009¤	0.033	0.017	0.013	0.028	0.001¤	0.012¤	0.001¤	0.003¤	0.003¤	0.009¤	0.012	0.011
12	0.004¤	0.020	0.006¤	0.013	0.013	0.002¤	0.068	0.004¤	0.001¤	0.005¤	0.008¤	0.013	0.019
13	0.007¤	0.014	0.007¤	0.006¤	0.050	<LOD	0.006¤	0.003¤	0.0004¤	0.0006¤	0.032	0.013	0.016
14	0.042	0.018	0.009¤	0.013	0.011¤	0.004¤	0.010¤	0.0007¤	0.003¤	0.0009¤	0.011¤	0.011¤	0.012
15	0.005¤	0.024	0.010¤	0.011¤	0.053	0.001¤	0.008¤	0.001¤	0.001¤	0.001¤	0.014	0.012	0.015
Stratum Corneum (Total Tape Strips)	0.198	0.402	0.268	0.199	0.453	0.049	0.510	0.065	0.066	0.067	0.255	0.230	0.166
Flange	0.524	0.315	0.260	0.838	2.76	0.372	0.306	0.337	0.163	0.076	0.304	0.568	0.75
Dermis	0.340	1.29	0.464	3.50	0.831	1.83	1.38	0.956	0.618	0.476	1.18	1.17	0.897
Skin Membrane^	0.864	1.60	0.724	4.34	3.59	2.20	1.68	1.29	0.781	0.552	1.48	1.74	1.22
Remaining Epidermis	0.412	1.04	0.347	2.70	0.947	1.22	0.909	0.907	0.443	0.260	0.605	0.890	0.679
Receptor Fluid	10.5	25.1	11.7	17.4	11.3	14.4	7.84	10.1	11.7	8.15	16.3	13.1	4.99
Systemically available*	11.8	27.7	12.8	24.4	15.9	17.8	10.4	12.3	12.9	8.96	18.4	15.8	5.91
TOTAL	96.8	95.3	99.6	97.6	97.1	98.6	98.3	96.5	101	98.3	99.1	98.0	1.58

^Skin Membrane = Sum of Flange and Dermis

*Systemically available = Sum of Skin Membrane, Receptor Fluid and Remaining Epidermis (values below the double line)

¤ = Less than LOQ (Limit of Quantification)

Highlights

- Dermal absorption of testosterone has been assessed using OECD 428 test guideline
- Mass balance distribution of testosterone was compared in both human and pig skin
- Absorption profiles were similar but pig skin was more permeable to testosterone
- Resected skin is now a widely accepted model to predict systemic exposure in man

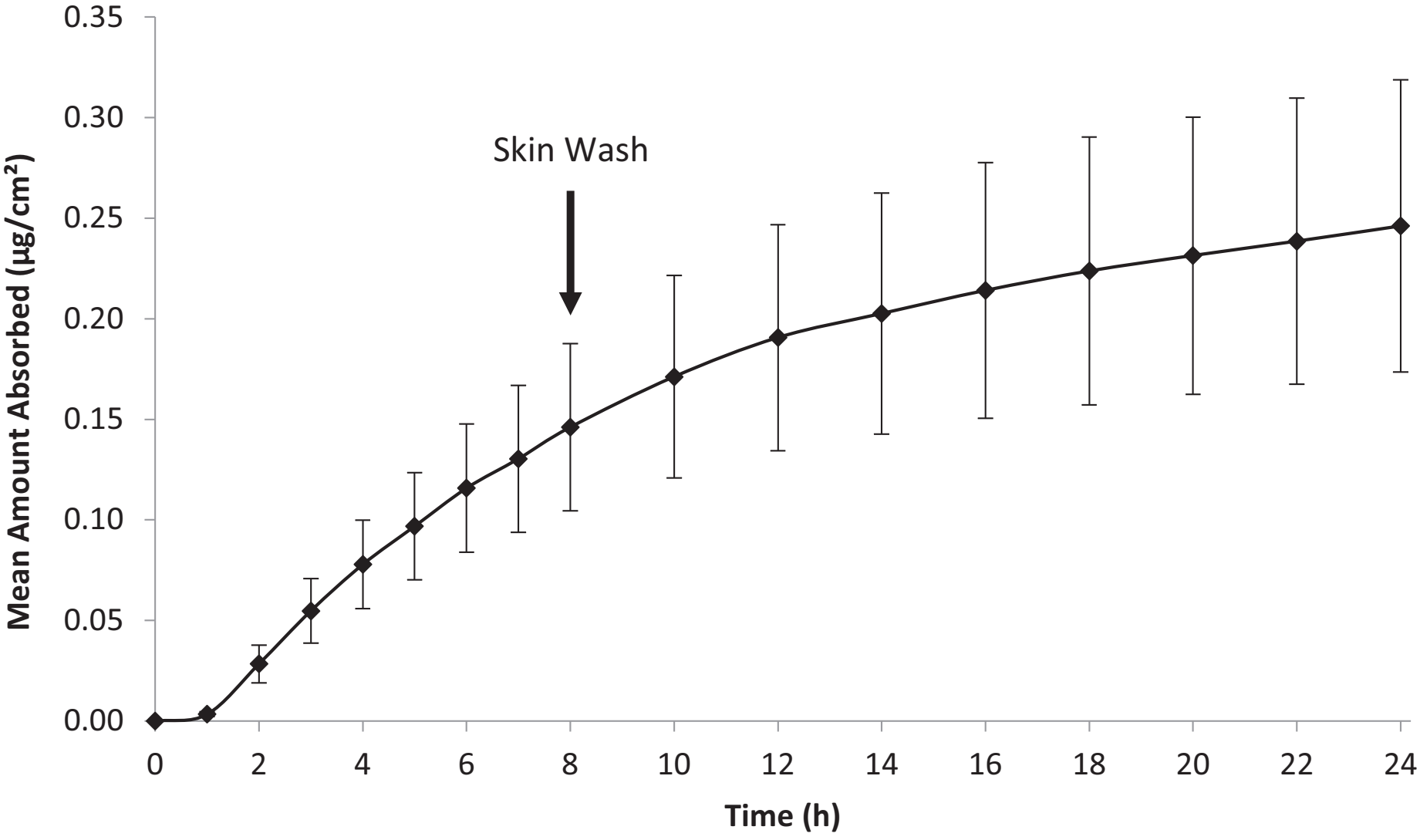
Mean Cell Data (± SEM; n = 8)
Mean Amount Absorbed (µg/cm²)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Skin Wash
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (h)

Figure 1

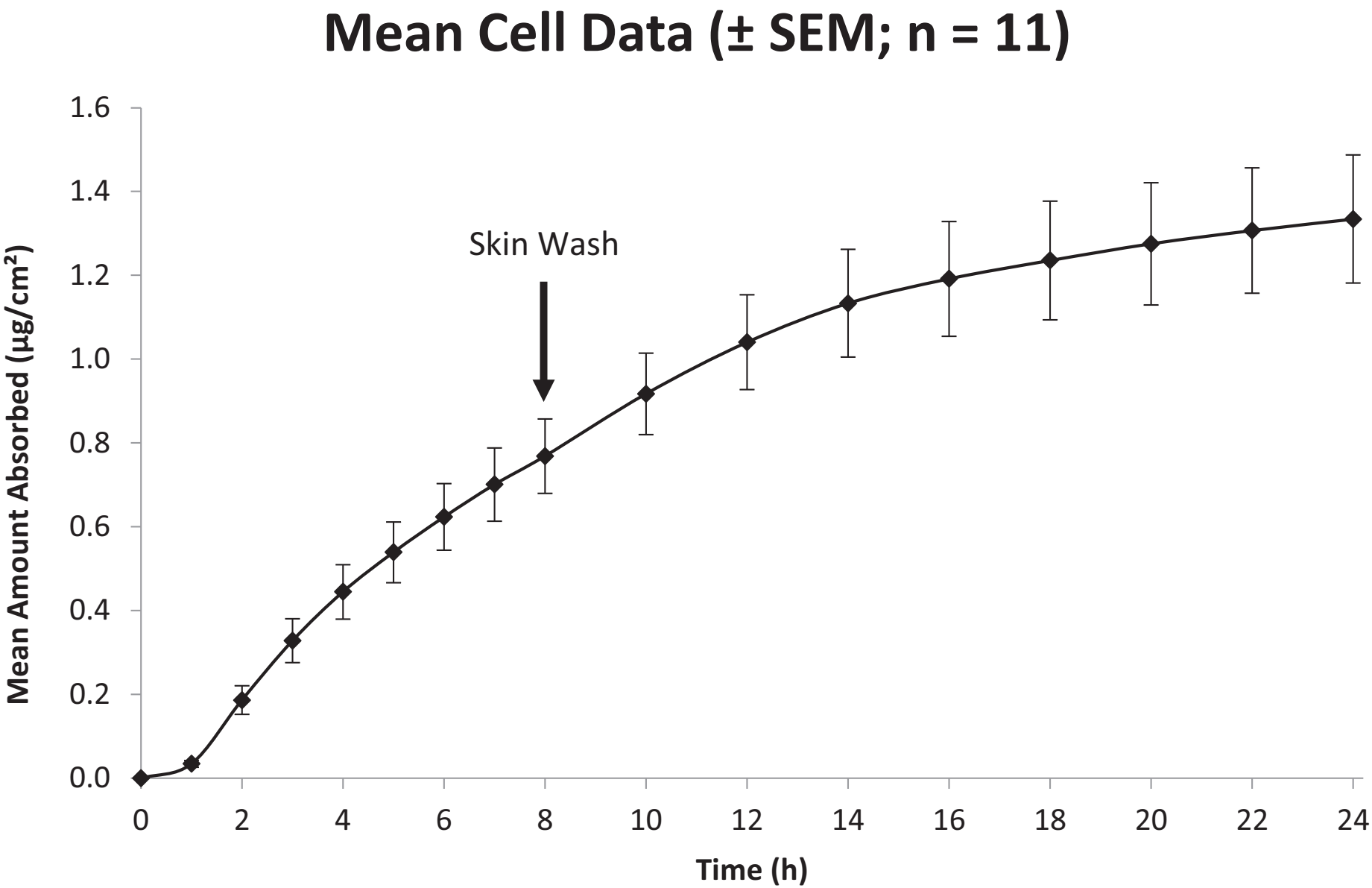
Mean Cell Data (± SEM; n = 11)
Mean Amount Absorbed (µg/cm²)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Skin Wash
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (h)

Figure 2