Topical bioavailability of diclofenac from locally-acting, dermatological formulations

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Running title: Topical bioavailability of diclofenac

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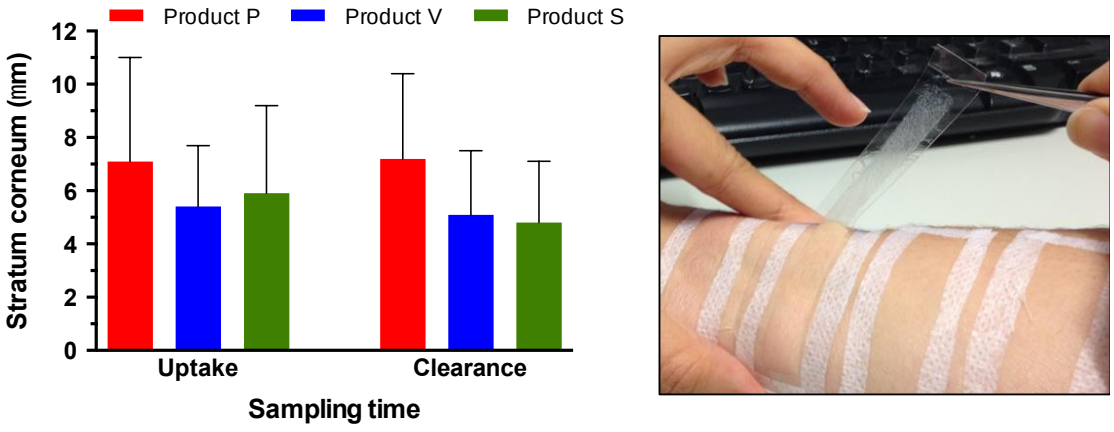


Key Words

Diclofenac, skin, topical bioavailability, topical bioequivalence, dermato-pharmacokinetics

Abbreviations

A	area of skin
ANOVA	analysis of variance
DMSO	dimethyl sulfoxide
FDA	U.S. Food & Drug Administration
HPLC	high performance liquid chromatography
IVPT	*in vitro* skin permeation test
$J_{in\ vitro}$	average flux of drug into the receptor chamber *in vitro* over the designated time interval
$J_{in\ vivo}$	average flux of drug from the SC into the underlying tissue *in vivo* over the duration of the clearance Δt
$M_{Cl,23h}$	mass of drug in the SC at 23 hours *in vivo* (Clearance for 17 h)
M_R	cumulative mass of drug entering the receptor solution from skin *in vitro*
$M_{R,jh}$	cumulative mass of drug entering the receptor solution after j hours *in vitro* (*j* = 6, 8, 23 and 24 h as designated)
$M_{S,n}$	mass of drug in the n^{th} sample vial
$M_{Up,6h}$	mass of drug in the SC at 6 hours *in vivo* (Uptake)
P	Pennsaid® solution 2%
Q_R	flow rate of receptor solution *in vitro*
Q1	same qualitative composition
Q2	same quantitative composition
S	Solaraze® gel 3%
SC	stratum corneum
t_n	time between drug application and the end of the n^{th} sampling interval
TEWL	transepidermal water loss
V	Voltaren® gel 1%
V_R	volume of receptor chamber
$V_{S,n}$	volume of solution in the n^{th} sample vial
Δt	duration of the clearance period

ABSTRACT

Assessment of the bioavailability of topically applied drugs designed to act within or beneath the skin is a challenging objective. A number of different, but potentially complementary, techniques are under evaluation. The objective of this work was to evaluate *in vitro* skin penetration and stratum corneum tape-stripping *in vivo* as tools with which to measure topical diclofenac bioavailability from three approved and commercialized products (two gels and one solution). Drug uptake into, and its subsequent clearance from, the stratum corneum of human volunteers was used to estimate the input rate of diclofenac into the viable skin layers. This flux was compared to that measured across excised porcine skin in conventional diffusion cells. Both techniques clearly demonstrated (a) the superiority in terms of drug delivery from the solution, and (b) that the two gels performed similarly. There was qualitative and, importantly, quantitative agreement between the *in vitro* and *in vivo* measurements of drug flux into and beyond the viable skin. Evidence is therefore presented to support an *in vivo – in vitro* correlation between methods to assess topical drug bioavailability. The potential value of the stratum corneum tape-stripping technique to quantify drug delivery into (epi)dermal and subcutaneous tissue beneath the barrier is demonstrated.

1 Introduction

The assessment of drug bioavailability following oral administration is a relatively straightforward exercise based on the reasonable assumption that the blood/plasma/serum level profile of the active moiety is a reliable surrogate for that at the site of pharmacological action. As a result, establishing the bioequivalence between oral dosage forms generally involves a standard, validated protocol (involving *in vivo* pharmacokinetic studies) that is recognized by regulatory authorities all over the world.

However, in the case of drug products applied to treat local disease either within, or directly below, the skin, the measurement of bioavailability – and, by extrapolation, bioequivalence - is more complicated (Shah et al., 2015). Here, the relationship between drug concentration at the site of action and that in the systemic compartment is less clear, and the physical measurement of either of those concentrations has proved challenging (if not impossible).

As a result, there is an ongoing effort to develop methodologies with which to evaluate the topical bioavailability and bioequivalence of locally-acting dermatological products (Herkenne et al., 2008; Lehman et al., 2011; Shah et al., 2015). This is particularly important for generic topical products for which, in most cases, the route to regulatory approval is uniquely via expensive, onerous and sometimes quite insensitive clinical outcome studies (Shah et al., 2015). Several approaches for the determination of topical bioavailability and bioequivalence are under investigation, including the use of *in vitro* (human) skin permeation tests, microdialysis (or microperfusion), stratum corneum (SC) tape-stripping, and non-invasive optical/spectroscopic techniques (Yacobi et al., 2014; Raney et al., 2015; Bodenlenz et al., 2017). While it seems unlikely that a single, 'gold-standard' method will be sufficient to uniquely evaluate the bioavailability/bioequivalence of topical products, there is a growing recognition that the rational combination of selected techniques can provide a "weight of evidence" support for such an assessment. The choice of tests would depend, for example, on factors such as the complexity of the drug product (Chang et al., 2013), as well as the drug's potency (and potential for systemic side effects), and site of action. For each potential approach, a robust consideration of practical methodological detail, including the number of replicates/subjects required to power a study and appropriate acceptance criteria, will ultimately be required to inform regulatory decision-making.

The aim of the work presented here is to demonstrate a proof-of-concept for the use of complementary methods in topical bioavailability/bioequivalence assessment. Specifically, the SC tape-stripping approach *in vivo* has been used together with *in vitro* skin permeation to compare three marketed diclofenac products, which are approved for different therapeutic indications and are not considered bioequivalent. One formulation, Solaraze® (diclofenac topical gel 3%), is used to treat actinic keratosis, while the other two, Voltaren® (diclofenac topical gel 1%) and Pennsaid® (diclofenac topical solution 2%), are for pain relief in particular forms of arthritis.

SC tape-stripping was the subject of a (now withdrawn) U.S. Food & Drug Administration (FDA) guidance (U.S. FDA, 1998) and involves collecting the outermost skin layer (i.e., the SC) using adhesive tapes post-application of a drug-containing formulation; subsequently, the drug in the SC can be extracted and quantified. Recently, tape-stripping results, from experiments using modified (Parfitt et al., 2011) and improved (N'Dri-Stempfer et al., 2009) protocols, correctly mirrored the established bioequivalence of topical anti-fungal creams (the site of action of which, naturally, is the SC itself) using clinical end-point studies. However, there remains an open question as to whether SC tape-stripping is a useful (or even meaningful) method to assess the bioavailability and bioequivalence of topical drug products which are designed to elicit their effects either within the viable epidermis/dermis or, in the case of pain relief induced by diclofenac, for example, in the subcutaneous tissue beneath the site of application.

Consequently, it was decided to compare the results from SC tape-stripping *in vivo* with data from *in vitro* skin permeation experiments. Specifically, using the three diclofenac products, measurements from the optimized "uptake and clearance" SC tape-stripping protocol (as reported in a study with econazole nitrate creams (N'Dri-Stempfer et al., 2009)) were correlated with percutaneous fluxes determined in conventional *in vitro* Franz diffusion cell experiments. The hypothesis tested, therefore, was that drug "clearance" from the SC must reflect 'input' into the viable skin tissue and beyond, assuming that the SC is the rate-limiting barrier; i.e., into the subcutaneous space (or, in the case of an *in vitro* skin permeation test, the receptor solution of the diffusion cell).

2 Materials & methods

2.1. Materials

The formulations tested (Table 1) were Pennsaid® (diclofenac topical solution 2%) (Mallinckrodt Brand Pharmaceuticals, Inc., Staines-upon-Thames, UK), Voltaren® (diclofenac topical gel 1%) (Novartis, Basel, Switzerland) and Solaraze® (diclofenac topical gel 3%) (PharmaDerm, Princeton, NJ, USA). Diclofenac sodium, solvents and HPLC reagents were from Sigma (Gillingham, UK).

Table 1: Diclofenac formulations tested.

Product	Code	Indication	Components
Pennsaid 2% solution	P	Osteoarthritis of the knee	Diclofenac sodium, dimethyl sulfoxide, ethanol, water, propylene glycol, hydroxypropyl cellulose
Voltaren 1% gel	V	Joint pain	Diclofenac sodium, carbomer homopolymer Type C, cocoyl caprylocaprate, fragrance, isopropyl alcohol, mineral oil, polyoxyl 20 cetostearyl ether, propylene glycol, water, strong ammonia solution
Solaraze 3% gel	S	Actinic keratoses	Diclofenac sodium, hyaluronate sodium, benzyl alcohol, polyethylene glycol monomethyl ether, water

Abdominal pig skin was obtained from a local abattoir, dermatomed (Zimmer®, Hudson, OH, USA), to a nominal thickness of 750 µm, frozen within 24 hours of slaughter, and thawed before use.

2.2. Stratum corneum (SC) tape-stripping experiments

The protocol was approved by both the Research Ethics Approval Committee for Health at the University of Bath, and the FDA's Research Involving Human Subjects Committee. The approach closely followed a previous *in vivo* study using econazole (N'Dri-Stempfer et al., 2009); specifically, the mass of drug in the SC at one 'uptake' and at one 'clearance' time point was measured. Fourteen healthy volunteers (8 female, 6 male, mean age 28 ± 8 years), who met the study inclusion criteria (Table 2), participated in the study having given their informed consent. The test site was the volar forearm, at least 5 cm above the wrist and a minimum of 0.5 cm below the bend in the arm at the elbow; for volunteers with significant hair growth in the test region, the skin was shaved using a new disposable razor at least 24 hours before the study began.

On the first day of the experiment, both arms were washed (Carex Complete, Cussons, Manchester, UK) and dried then left for 1 hour to allow skin hydration to return to normal. This procedure ensured that the starting skin condition was, as close as possible, the same for all volunteers. Immediately before application of formulations, at a forearm site away from those to be treated, two tape-strips were taken to provide drug-free samples of SC to act as controls for the

analytical method. In addition, at the skin sites to be treated, the baseline (i.e., intact, unstripped skin) transepidermal water loss (TEWL) rate was measured (AquaFlux, Biox Systems Ltd., London, UK).

Six self-adhesive, foam padding frames (Pressure Point Foam Padding, Scholl, Slough, UK), with internal dimensions of 1.5 x 5.5 cm, were applied to each arm; as the width of the frame was 0.8 cm, the minimum distance between the edges of the treated skin sites was 1.6 cm. There were duplicate application sites for each product on both arms of each volunteer. Using a cotton bud (Johnson & Johnson, Berkshire, UK) to spread the formulation over the treatment area, products P and V were applied at 10 mg/cm^2, while product S was applied at 20 mg/cm^2, reflecting the respective recommended use levels. The exact amount applied was determined gravimetrically.

During the 6-hour uptake period, the sites were not occluded, but were protected by a plastic mesh (Ultra-stiff Plastic Canvas, 7 mesh, Darice®, Strongsville, OH, USA) affixed to the skin by Mefix® tape (Molnlycke, Lancashire, UK); the depth of the padding material used for the frame was approximately 3 mm, so the mesh was not in contact with the formulation. Volunteers were prohibited from bathing during the study or participating in strenuous activity, but otherwise pursued their normal lifestyle.

Table 2: Inclusion criteria for participants.

Healthy; aged between 18-72 years old
Male or non-pregnant and non-breastfeeding female
Any ethnic background
Willing to provide basic information (i.e., age, height, weight, health, pregnancy status, gender and ethnicity and handedness)
No simultaneous participation in another clinical trial or cosmetic study
No skin infection, chronic skin disease, hereditary skin disorders or any skin inflammatory conditions
No excessive pigmentation, tattoos, hair, moles, skin defects, sunburn, or blemishes
Not a current smoker, and having BMI < 30 kg/m^2
No alcohol for 24 hours prior to the start of the study
Not using any topical drugs at the test site area
No prescription medication during the period 0-30 days or over-the-counter medication 0-5 days before entry to the study (with the exception of oral contraceptives)
No strenuous exercise during the study period
No sunbathing or exposure of arms to sun or UV light during participation and/or during the week after
No previous adverse reaction to diclofenac or to the other ingredients in the formulations tested, to medical dressings, or to adhesive tapes
Length of forearm ≥ 24 cm

After the 6-hour application of the products, formulation remaining on the skin was removed from all 12 sites using (a) one dry tissue (Wypall, Kimberly Clarke, Kent, UK), and (b) two 70% isopropyl alcohol wipes (Sterets®, Molnlycke, Lancashire, UK). The dry wipe was included when it was

observed that, after a 6-hour application of product S, residual solid material ('flakes') were visible on the treated skin. The cleaned sites on one arm were then designated as "uptake", while those sites on the other were designated as "clearance".

2.2.1 Uptake sites, tape-stripped immediately post 6 hours of product application

Templates (Scotch® Book Tape, 3M, St. Paul, MN, USA), with internal dimensions of 1 x 5 cm, were prepared and adhered to each of the treated sites leaving open the skin area to be stripped. SC at the 6 uptake sites was then detached via sequential application and removal of adhesive tape-strips (1.5 x 6.5 cm, cut from Scotch® Book Tape). To ensure both the comfort of the volunteers, and that a significant fraction of the SC was removed by the tape-stripping procedure (N'Dri Stempfer et al., 2009), TEWL was measured intermittently during tape-stripping (Kalia et al., 1996; Kalia et al., 2000) until either (i) the rate of water loss reached 60 $g \cdot m^{-2} \cdot h^{-1}$, or exceeded 6-fold the baseline, pre-treatment control value, or (ii) 30 tape-strips had been removed.

2.2.2 Clearance sites, tape-stripped 23 hours after product application

Immediately after cleaning, the clearance sites were demarcated using Mefix® tape (Molnlycke, Lancashire, UK), without encroaching on the treated areas. The entire volar forearm was then covered with light gauze (Boots, Nottingham, UK) to protect the sites overnight. Then, the following day, 23 hours after product application (and 17 hours after product removal from skin surface), the 6 clearance sites were tape-stripped in exactly the same way as performed at the uptake sites.

2.2.3 Processing of the tape-strips

All tapes were weighed (Microbalance SE-2F, precision 0.1 µg; Sartorius AG, Goettingen, Germany) before and after stripping to determine the mass of SC removed; tapes were first discharged of static electricity (R50 discharging bar and ES50 power supply Eltex Elektrostatik GmbH, Weil am Rhein, Germany) to facilitate the weighing procedure. Diclofenac was extracted from the first two tapes individually but, thereafter, tape-strips from the deeper SC were analysed in groups of up to 5 to ensure, as far as possible, that analytical sensitivity was maintained. No tapes were discarded. Drug was extracted from the tape-strips with 3 mL of 4:1:1 methanol:hexane:ethyl acetate per sample, using sonication for 1 hour at 55°C followed by shaking overnight at 37°C. One mL of each sample was evaporated and re-suspended in 1mL of mobile phase and then vortexed for 3 seconds. Samples were filtered (0.45 µm nylon membrane, Labhut, Maisemore, UK) before analysis by high-performance liquid chromatography (HPLC).

2.3. In vitro skin permeation test (IVPT) studies

The formulations were applied to excised abdominal porcine skin mounted in diffusion cells with the dermal side in contact with a physiological buffer. The skin was not occluded and the formulations were not removed from the skin. The two sets of IVPT studies performed used the same receptor solution, which was stirred with a magnetic bar, of phosphate-buffered saline (pH 7.4) with (set 1) or without (set 2) sodium azide 0.01% w/v.

2.3.1. IVPT set 1

Vertical flow-through cells (Laboratory Glass Apparatus, Berkeley, CA, USA) with an exposed skin area of 3.14 cm^2 were used. Formulations were applied using the flat base of a 2 mL glass vial, and the mass of product applied was determined by simple weight difference. Four replicates were performed per formulation using skin from donor 'Pig A'. The receptor solution volume was ~7.7 mL, and was thermostatted via recirculating water maintained at 37°C. The receptor solution flow rate was ~1 mL/h (the average for each cell was measured) and samples were collected on a fraction collector every 4 hours.

2.3.2. IVPT set 2

Static, Franz cells (Permegear, Hellertown, PA, USA) with an exposed skin area of 2.01 cm^2 and a receptor volume of ~7.4 mL were used. Formulations were applied using a cotton bud (i.e., similar to the *in vivo* tape-stripping experiments), and the mass of product applied was again determined by weight difference. Eight replicates were performed per formulation, 4 using skin from donor 'Pig B', and 4 with skin from donor 'Pig C'. Throughout the experiment, the diffusion cells remained in an oven at 32°C and 40% relative humidity, except for brief excursions when the receptor solution was sampled (1 mL) at 6, 8, 10, 12, 23, 26 and 30 hours. The concentrations of diclofenac measured in the receptor phase samples were never more than 2 µg/mL, i.e., far below the aqueous solubility of the drug at pH 7.4 (which is, in fact, about 10^3-fold higher; Maitani et al., 1993), therefore ensuring that sink conditions were maintained throughout the experiment.

2.4. High-performance liquid chromatography (HPLC)

Diclofenac was quantified by HPLC (Summit, Dionex, Camberley, UK) with UV detection (284 nm). A mobile phase of 65:35 methanol:0.2% triethylamine (adjusted to pH 2.85 with phosphoric acid) was pumped (1mL/min) through a 250 x 4.6 mm Kinetex C18 column (Phenomenex, Macclesfield, UK). Retention time was ~10 minutes. Injection volume was 50 µL; limits of quantification and detection were 0.03 and 0.01 µg/mL, respectively.

2.5. Data analysis

2.5.1. SC tape-stripping

The thickness of SC removed on each tape was calculated from the weight and area sampled, assuming a tissue density of 1 g/cm^3 (Anderson & Cassidy, 1973).

At both uptake and clearance, the thickness of stratum corneum removed, the number of tapes used, and final TEWL value measured were compared across the 3 products using a repeated measures ANOVA followed by Bonferroni post-tests. In addition, for each product, the same parameters were compared using a paired t-test. The geometric mean of the mass of drug extracted (in the two duplicate sites) from the tape-strips was calculated for each volunteer; these values were then averaged (arithmetic mean) across n = 14, for each time point and each product.

The average flux of drug from the SC into the underlying tissue between the uptake and clearance time points ($J_{in\ vivo}$) was calculated for each formulation and for each volunteer:

$$J_{in\ vivo} = (M_{Up,6h}/A - M_{Cl,23h}/A)/\Delta t \qquad \text{(Eq. 1)}$$

where $M_{Up,6h}$ is mass of drug in the SC at 6 hours after drug application, $M_{Cl,23h}$ is mass of drug in the SC at 23 hours, A is the sampled skin area, and Δt is the duration of the clearance period, i.e., 17 hours.

Further, assuming a first-order clearance of the drug from the SC, then the associated rate constant, k, is given by:

$$k = -\ln(M_{Cl,23h}/M_{Up,6h})/\Delta t = -\ln(M_{Cl,23h}/M_{Up,6h})/(17\ h) \qquad \text{(Eq. 2)}$$

Note that k is a rate constant averaged over the clearance period of 17 h. It may not necessarily have the same value for shorter or longer periods of clearance.

2.5.2. IVPT set 1

The flux of drug into the receptor solution from the skin (J_n) during the n^{th} sampling time interval was calculated using the following equation (Touraille et al., 2005):

$$J_n = \frac{Q_R}{V_R}\left[\frac{M_{S,n}/A}{V_{S,n}/V_R + \exp(-t_n Q_R/V_R)\cdot\{1-\exp(V_{S,n}/V_R)\}}\right] \qquad \text{(Eq. 3)}$$

where: A = treated skin area, $M_{S,n}$ = mass of drug in the n^{th} sample vial, t_n is the time between drug application and the end of the n^{th} sampling interval, Q_R = volumetric perfusion rate of receptor solution, V_R = volume of receptor chamber, and $V_{S,n}$ = volume of the n^{th} sample. The cumulative mass of drug into the receptor solution at time t_m was calculated by numerical integration of J_n using the trapezoid rule (where J_0 is assumed to be zero):

$$\frac{M_{R,m}}{A} = \sum_{n=1}^{m} \frac{(t_n - t_{n-1})}{2}(J_n + J_{n-1}) \qquad \text{(Eq. 4)}$$

The apparent average flux of drug into the receptor chamber between 8 and 24 hours ($J_{in\ vitro}$) was then calculated for each cell:

$$J_{in\ vitro} = (M_{R,24h}/A - M_{R,8h}/A)/\Delta t = (M_{R,24h}/A - M_{R,8h}/A)/16h \qquad \text{(Eq. 5)}$$

where $M_{R,8h}$ and $M_{R,24h}$ are the cumulative mass of drug permeated into the receptor chamber after 8 hours and 24 hours, respectively.

2.5.3. IVPT set 2

The amount of drug permeated at each time interval was calculated from the measured concentration in the receptor solution, taking into account that removed in each sampling. The flux of drug into the receptor chamber between 6 and 23 hours was then calculated for each cell:

$$J_{in\ vitro} = (M_{R,23h}/A - M_{R,6h}/A)/17h \qquad \text{(Eq. 6)}$$

Data obtained using skin from the two pig 'donors' were pooled, and $J_{in\ vitro}$, $M_{R,6h}$ and $M_{R,23h}$ were then compared across the 3 products using a one-way ANOVA followed by Bonferroni post-tests. Statistical significance was again set at $p < 0.05$.

2.5.4. Statistics

The cumulative mass into the receptor (M_R), and the flux (J), for the SC tape-stripping and IVPT experiments, as well as the mass in the SC for the *in vivo* tape-stripping, were compared across the 3 products using a repeated measures ANOVA followed by Bonferroni post-tests. Also, for each product in the SC tape stripping experiment, $M_{Up,6h}$ was compared to $M_{Cl,23h}$ using a paired t-test. In all the comparisons undertaken, statistical significance was set at $p < 0.05$. Reported confidence intervals were calculated using the Student's T-distribution for the sample size and the sample standard deviation.

3 RESULTS

3.1. SC tape-stripping experiments

This component of the study was performed in 14 healthy volunteers and assessed the method's ability to distinguish between drug products that were not expected to be bioequivalent. No adverse events were recorded, and all volunteers completed the study. The clinically relevant doses of formulation applied were (arithmetic mean ± standard deviation) 9.9 ± 0.7, 10.3 ± 0.6 and 19.4 ± 1.6 mg/cm^2 for products P, V and S, respectively. Table 3 summarizes information about the collection of SC from the treated sites at both uptake and clearance times. An adequate fraction of the SC was removed (total SC thickness on the ventral forearm has been reported to be 10.9 ± 3.5 µm (Kalia et al., 2000)).

Table 3: SC collection from the treated sites – numbers of tapes used, thickness of SC removed and final TEWL measured when tape-stripping stopped. Arithmetic mean of within-subject duplicates ± standard deviation across all 14 subjects. Pairs of superscript letters indicate statistical differences ($p < 0.05$).

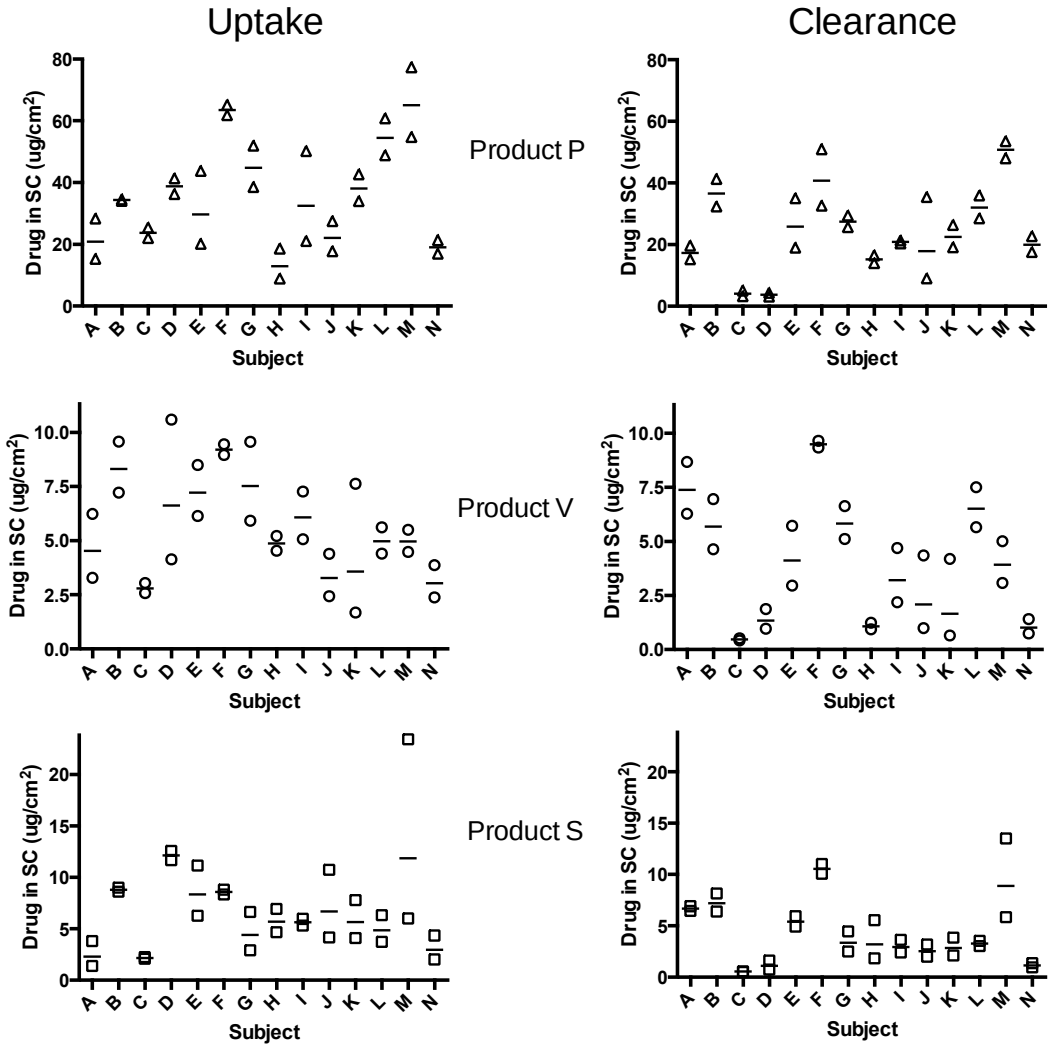
Product	P		V		S	
	Uptake	Clearance	Uptake	Clearance	Uptake	Clearance
Number of tapes used	17 ± 10^{a,b}	23 ± 9^{c,d}	30 ± 0^{a}	30 ± 0^{c}	30 ± 1^{b}	30 ± 0^{d}
SC thickness removed (µm)	7.1 ± 3.9^{e}	7.2 ± 3.2^{f,g}	5.4 ± 2.3^{e}	5.1 ± 2.4^{f}	5.9 ± 3.3	4.8 ± 2.3^{g}
Final TEWL* (g·m^{-2}·h^{-1})	63.1 ± 21.1^{h,i}	51.8 ± 14.0^{j,k}	32.2 ± 19.5^{h}	24.5 ± 16.2^{j}	27.8 ± 13.6^{i}	21.5 ± 9.8^{k}

*Mean baseline TEWL was measured to be 11.6 ± 3.5 (g·m^{-2}·h^{-1}). There was no significant difference between the values determined at the three sets of sites treated with the three different products (i.e., SC integrity pre-treatment was consistent at all skin sites used).

A clear formulation-specific effect on SC removal was observed. More SC was detached, using fewer tapes, from sites treated with product P relative to those exposed to products V or S. At sites to which product P had been applied, it was frequently observed that, within discrete parts of the sampled area, several layers of corneocytes appeared to have been removed with a single tape (a phenomenon that has been previously reported with an acyclovir formulation (Russell & Guy, 2012)). This effect was seen at skin sites used for both uptake and clearance measurements; i.e., independent of whether tape-stripping was performed immediately post-formulation removal or 17 hours later.

Diclofenac was easily quantified in the SC, with less than 1% of the samples analysed having amounts below the limit of quantitation. Figure 1 shows the total drug mass recovered from the SC for each volunteer at uptake and clearance times. Some variability between duplicate measurements (2 sites with same product at the same time point in the same person) was

observed, and may be due to differences between the skin sites, or variability in application of the formulations, or variability in the efficiency of removing unabsorbed formulation at the end of the uptake period. Inter-subject variability in dermal drug absorption was observed as expected, highlighting the importance of comparing products within-subject to increase the method's sensitivity to detect differences between products.



<u>Figure 1</u>: Mass of diclofenac recovered from each skin site, with duplicate determinations performed for each product and each time point for all subjects (n = 14). Horizontal lines are the geometric means of the 2 duplicate values (shown by the symbols).

There was a clear distinction, as seen in Figure 1 (note the different y-axis scales) and Table 4, in the amounts of diclofenac taken up into the SC from the 3 products. The mass of drug recovered from the SC was significantly greater ($p < 0.05$) after application of product P than that post-treatment with either product V or product S, at both uptake and clearance. However, the results for products V and S were much closer.

For all products, the mass of drug in the SC at uptake was significantly higher than that at clearance, indicating that measurable transfer of diclofenac into the viable skin (and presumably

beyond) had occurred. The average, relative depletion of drug from the SC during the clearance phase (33%, 31% and 33%, respectively for products P, V and S) was similar to that observed previously for econazole (N-Dri'Stempfer et al., 2009); for 26 of the 42 pairs of values, ≥25% of the drug in the SC at uptake was cleared over the next 17 hours.

Table 4: Mass of diclofenac recovered from the SC after uptake and clearance ($M_{Up,6h}$ and $M_{Cl,23h}$, respectively), the deduced drug flux from the SC into the underlying viable tissue during the clearance period ($J_{in\ vivo}$), and the first-order rate constant (k) describing clearance from the SC. Arithmetic mean ± 90% confidence interval (n = 14). Pairs of superscript letters indicate statistical difference.

Product	P	V	S
$M_{Up,6h}/A$ (µg·cm^{-2})	36 ± 7.7^{a,b,g}	5.5 ± 1.0^{a,h}	6.4 ± 1.5^{b,i}
$M_{Cl,23h}/A$ (µg·cm^{-2})	24 ± 6.2^{c,d,g}	3.8 ± 1.3^{c,h}	4.3 ± 1.4^{d,i}
$J_{in\ vivo}$ (ng·h^{-1}·cm^{-2})	694 ± 312^{e,f}	97 ± 59^{e}	128 ± 93^{f}
k (h^{-1})	0.030 ± 0.020	0.036 ± 0.019	0.033 ± 0.022

Figure 2 presents the drug concentration *versus* SC depth profiles post-application of the 3 products at uptake and clearance. In this case the y-axis scales are the same for the three products and the profiles again illustrate clearly the enhanced delivery of diclofenac from product P relative to the other two formulations. Visually inspecting profiles like these serves as a useful check on the quality of the results obtained, for example, to detect very clear outliers in the data or (if the profile appears 'truncated') to indicate insufficient sampling of drug from the deeper SC.

Although there is no reason to consider the three products evaluated in this study as bioequivalent, it is possible to illustrate how the results obtained may be used to address this issue. Table 5 shows the results of a bioequivalence assessment of the 3 products. For each volunteer, the ratio of the mass of diclofenac in the SC (at uptake and clearance) was calculated between pairs of products. The log transformed ratios were then averaged across all participants and the 90% confidence interval calculated (US FDA, 2001). The average and 90% confidence intervals of the ratios (i.e., the inverse log transformation of the average and confidence intervals of the log transformed ratios) are in Table 5. Typically, if the confidence intervals fall within the range of 0.8 to 1.25, the two products are considered bioequivalent. As expected from the high-level evaluation of the data presented thus far, the results of this analysis are (a) that product P is conclusively inequivalent to both products S and V, and (b) that products S and V fail the equivalency test at the lower end of the range.

Table 5: "Bioequivalence" assessment between the 3 products. Values are inversed logs of the {log[mean ratio]} for product pairs (lower - upper 90% confidence interval) for n = 14 subjects.

Comparison	P to V	P to S	V to S
Uptake	6.27 (5.10 - 7.71)	5.69 (4.47 - 7.25)	0.91 (0.74 - 1.12)
Clearance	6.94 (5.16 - 9.32)	6.06 (4.82 - 7.62)	0.87 (0.70 - 1.09)

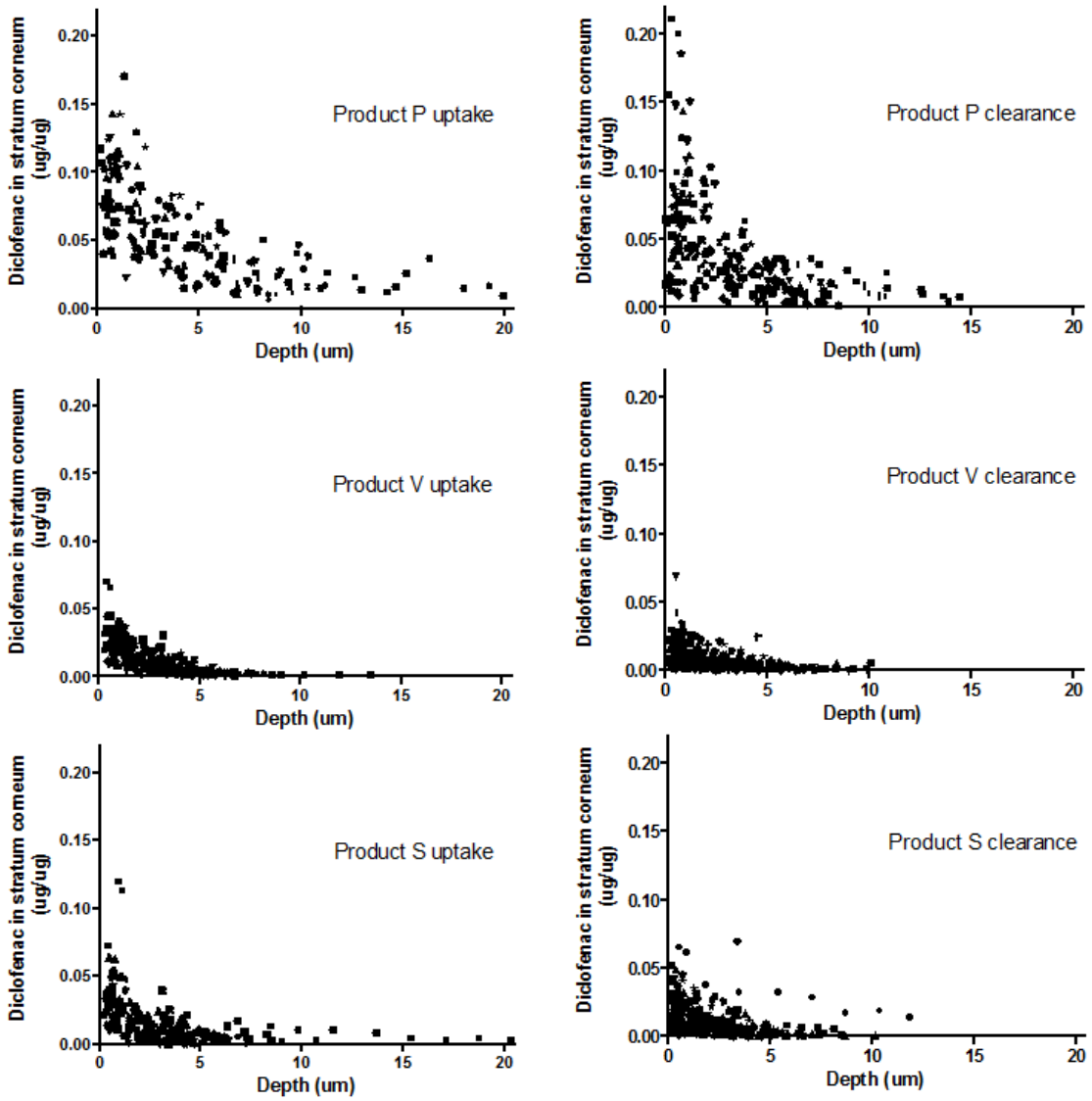


Figure 2: Diclofenac concentration versus SC depth profiles following application of 3 drug products and assessed after uptake and clearance; raw data (not averages of duplicate values) are plotted. Data from tape-strips with a very low mass of SC (<0.012 mg per group) have not been plotted. When tape-strips were grouped for drug analysis, the SC depth plotted on the x-axis represents the midpoint for that sample.

While this approach can assess the relative bioavailability of the drug from different products, the relevance of doing so for diclofenac, the site of action of which is clearly not the SC, may be questioned. For this reason, the uptake and clearance amounts of the drug in the SC were used to deduce an average flux ($J_{in\ vivo}$) during the 17 hours that elapsed between the two measurements (see Table 4). Although these values were associated with relatively large standard deviations (primarily because, for 3 out of 14 cases for each product, the observed difference between $M_{Up,6h}$ and $M_{Cl,23h}$ was indistinguishable from zero), the conclusion drawn from the results is clear: namely, that product P delivered significantly more drug both into *and* through the SC than either of products V and S; the performance of the latter two products, however (again, both in terms of diclofenac delivery into and across the SC), was not significantly different.

It is also noteworthy that, while the flux through the skin is much larger from product P than from products V and S, the estimated clearance rate constants (recalling that flux is proportional to rate constant multiplied by SC concentration) are not statistically different for the 3 products. This is completely consistent with the higher flux from product P resulting from the greater amount of diclofenac in the SC at the end of the uptake period (and not, for example, being due to a larger diffusivity).

3.2. IVPT experiments

The IVPT results from "set 1" and "set 2" experiments are summarized in Figure 3.

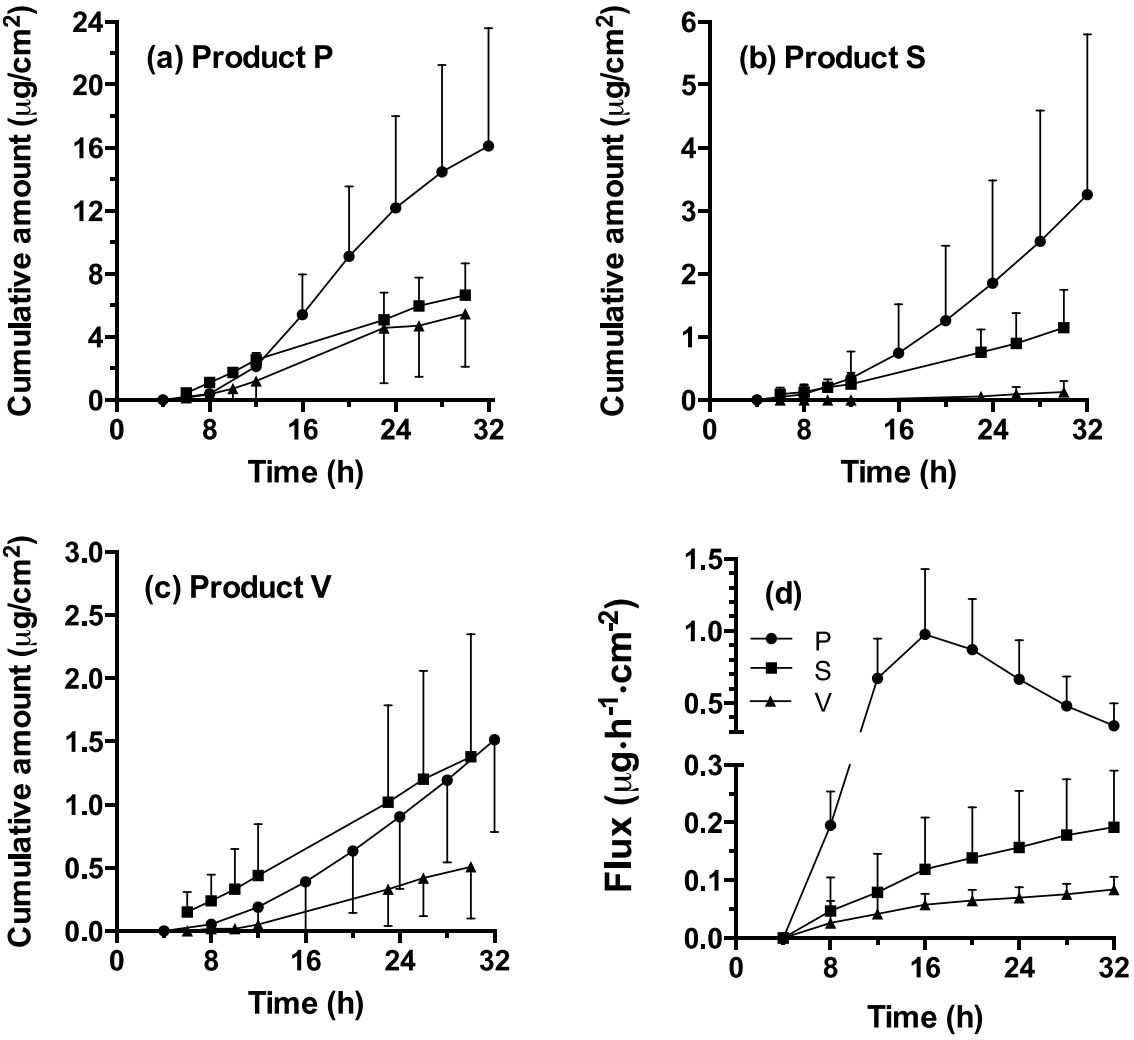
3.2.1. IVPT set 1

The clinically relevant doses of formulation applied were (arithmetic mean ± standard deviation) 10.6 ± 1.1, 10.3 ± 0.6 and 19.7 ± 0.4 mg/cm^2 for products P, V and S, respectively. The cumulative penetration of diclofenac into the receptor chamber of the diffusion cell after 8 and 24 hours, and the deduced flux across the skin from the 3 formulations are in Table 6. Statistical analysis of the results was entirely consistent with the *in vivo* data discussed above: each of the metrics, $M_{R,8h}$, $M_{R,24h}$ and $J_{in\ vitro}$, was significantly larger for product P than both products V and S, which were statistically indistinguishable. Panel (d) of Figure 3, which shows the permeation fluxes of diclofenac from the three formulations as a function of time, clearly reinforces these observations. The steady increase in flux over the 32-h experiment for products V and S differed from that for product P, which exhibited a maximum at about 16 h followed by a slow decline. The significant presence of DMSO in the composition of product P is undoubtedly the major reason behind the distinct behaviour of this formulation.

3.2.2. IVPT set 2

The doses of formulation applied were (arithmetic mean ± standard deviation) 10.0 ± 0.5, 9.4 ± 0.3 and 20.2 ± 1.1 mg/cm^2 for products P, V and S, respectively. This series of experiments was performed using skin from two additional pigs. The cumulative penetration of diclofenac into the

receptor chamber of the diffusion cell after 6 and 23 hours, and the deduced flux across the skin from the 3 formulations are in Table 7; individual results from the measurements made with skin from the different 'donors' and the combined dataset are shown.



<u>Figure 3</u>: Panels (a), (b) and (c) show cumulative diclofenac permeation from 3 topical products, as a function of time across porcine skin in vitro, from the "set 1" experiments (skin from pig A, circles) and the "set 2" study (skin from pigs B (squares) and C (triangles)). Panel (d) - diclofenac fluxes measured in the "set 1" experiments using flow-through diffusion cells. All data shown are the arithmetic mean and 90% confidence interval (n = 4 for each case).

Noticeable variability in drug permeation across skin from the different pigs was seen for products V and S; in contrast, for product P, excellent agreement for the two skin sources was observed. These observations could be related to a reduction of the skin barrier by product P that does not occur for products V and S. Nevertheless, despite this variability, and the procedural and data analytical differences used in the two sets of IVPT experiments (different diffusion cells, thermostatting method, sample times chosen for comparison), the results in "set 2" mirror exactly those seen in "set 1" and are consistent once more with those observed in the *in vivo* SC tape-

stripping study: each of the metrics, $M_{R,6h}$, $M_{R,23h}$ and $J_{in\ vitro}$ for product P was significantly greater than those for products V and S, which were statistically indistinguishable.

Table 6: Results from the in vitro penetration test "set 1" experiments. The data (arithmetic mean ± 90% confidence (n = 4)) were obtained using skin from pig A in flow-through diffusion cells. Pairs of superscript letters indicate statistical difference.

Product	**P**	**V**	**S**
$M_{R,8h}$/A (µg·cm^{-2})	0.39 ± 0.14^{a,b}	0.05 ± 0.09^{a}	0.09 ± 0.14^{b}
$M_{R,24h}$/A (µg·cm^{-2})	12 ± 5.8^{c,d}	0.90 ± 0.57^{c}	1.9 ± 1.64^{d}
$J_{in\ vitro}$ (ng·h^{-1}·cm^{-2})	737 ± 357^{e,f}	53 ± 30^{e}	110 ± 94^{f}

Table 7: Results from the in vitro permeation test "set 2" experiments. The data (arithmetic mean ± 90% confidence) were obtained using skin from pigs B (n = 4) and C (n = 4) in static diffusion cells. Pairs of superscript letters indicate statistical difference; n.d. = not detected. Values in parenthesis for product S excludes 2 samples for which no measureable permeation was observed.

Product	**Skin (n)**	**$M_{R,6h}$/A (µg·cm^{-2})**	**$M_{R,23h}$/A (µg·cm^{-2})**	**$J_{in\ vitro}$ (ng·h^{-1}·cm^{-2})**
P	Pig B (4)	0.45 ± 0.11	5.1 ± 1.7	274 ± 95
	Pig C (4)	0.16 ± 0.25	4.6 ± 3.5	260 ± 193
	Combined (8)	**0.30 ± 0.15**^{a,b}	**4.8 ± 1.5**^{c,d}	**267 ± 80**^{e,f}
V	Pig B (4)	0.15 ± 0.16	1.0 ± 0.78	51 ± 37
	Pig C (4)	<LOQ	0.33 ± 0.29	19 ± 16
	Combined (8)	**0.08 ± 0.08**^{a}	**0.67 ± 0.39**^{c}	**35 ± 19**^{e}
S	Pig B (4)	0.07 ± 0.11	0.75 ± 0.36	40 ± 15
	Pig C (4)	n.d.	0.07 ± 0.08 (0.13 ± 0.02, n = 2)	4.3 ± 4.5 (7.5 ± 1.2, n = 2)
	Combined (8)	**0.04 ± 0.05**^{b} (0.05 ± 0.07, n = 6)	**0.41 ± 0.28**^{d} (0.54 ± 0.33, n = 6)	**26 ± 17**^{f} (29 ± 16, n = 6)

3.3. *In vivo – in vitro* comparison

The *in vivo* and the *in vitro* experiments performed both permit an estimation of the flux of diclofenac into the viable skin tissue (and beyond) from the 3 products examined. Figure 4 graphically presents a summary of the results obtained. Importantly, the overall message delivered by the data was consistent, namely, the enhanced drug delivery from product P compared to the other two formulations, and the similarity between the V and S products. In addition, the fluxes deduced from the two distinct experimental approaches are generally

consistent, despite the different skin sources used and the variations in the *in vitro* protocols adopted.

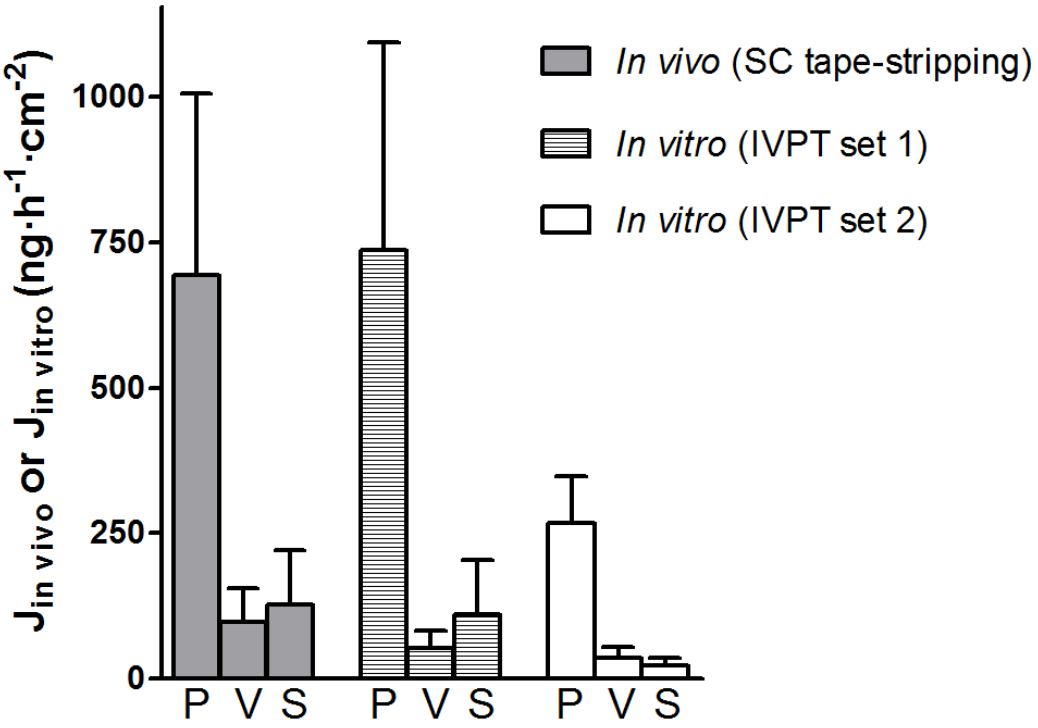


Figure 4: Comparison of $J_{in\ vivo}$ and $J_{in\ vitro}$ deduced from the SC tape-stripping study in human volunteers (n = 14) and the two sets of Franz diffusion cell measurements (n = 4 for set 1, n = 8 for set 2) using excised porcine skin (arithmetic mean + 90% confidence interval).

4 Discussion

The research described here has compared two methods, stratum corneum (SC) tape-stripping *in vivo* in man and *in vitro* skin permeation (using porcine tissue), as surrogate approaches with which to assess and compare a drug's topical bioavailability following application in different products. The experiments were undertaken using 3 marketed formulations containing diclofenac as the active pharmaceutical ingredient, with sites of action either in the viable skin below the SC barrier or deeper within the subcutaneous tissue. An important issue to address, therefore, was whether SC tape-stripping (which has already been shown capable of accurately evaluating the local bioavailability of anti-fungal drugs which act on or in the SC (N'Dri-Stempfer et al., 2009; Parfitt et al., 2011)) is able to provide a suitable metric to characterize the rate and/or extent of drug absorption to skin tissues beneath the SC. A further aim was to compare the *in vivo* results to those from the IVPT approach to examine whether a correlation and/or synergy might exist between the two methodologies.

SC tape-stripping *in vivo* has several advantages, including: (a) it can be performed in a minimally invasive way, in man; (b) the experiment is conducted with a fully functioning cutaneous microcirculation in operation that permits drug clearance from the skin to take place unimpeded; (c) uptake of drug into the SC can be assessed after much shorter time periods than are typically needed when using *in vitro* techniques; and (d) tissue viability throughout the experiment is assured.

Equally, the use of Franz diffusion cells *in vitro* with excised human (or other animal model) skin provides benefits that SC tape-stripping does not. For example, a dermal pharmacokinetic profile is available via frequent sampling of the receptor solution over the duration of an IVPT experiment, and is much less onerous, and far more practical, than performing tape-stripping at multiple time points; an IVPT experiment permits a direct evaluation of drug flux into and through the viable skin; and it is relatively straightforward, if required, to assay the drug levels in the epidermis/dermis at the end of the permeation experiment.

The SC tape-stripping experiments undertaken in this work provided three metrics with which to compare the 3 products considered: (i) the mass of drug in the SC after a 6-hour uptake period, (ii) the mass of drug in the SC after a subsequent 17-hour clearance period, and (iii) from the difference between these two values (per unit time), an estimate of the input rate or flux of the drug into the viable skin below the SC. Regardless of the metric chosen, the results were clear and statistically significant: the uptake and permeation of diclofenac from product P was much greater than that from either product S or product V, whereas the apparent bioavailability of the drug from products S and V was similar.

The higher performance of product P is most likely due to the presence (>40% w/w) of dimethyl sulfoxide (DMSO) in the formulation; neither product V nor product S contains this excipient.

DMSO is a well-known skin penetration enhancer, and is believed to exert its effect by altering protein (i.e., keratin) conformation and/or perturbation of intercellular lipid organization in the SC (Williams & Barry, 2012). DMSO itself is rapidly taken up into the SC and can also, through its excellent solvation properties, facilitate drug partitioning into the barrier (Williams & Barry, 2012). The similarity in the deduced clearance rate constants (Table 4), from the SC tape-stripping experiments using the three different products, suggests that the latter mechanism of DMSO enhancement is probably the dominant one for product P (although the easier removal of SC post-application of this product, and the extent to which TEWL was increased (Table 3), also suggest that some degree of barrier disruption was provoked).

Product P also produced a formulation-specific effect on SC collection (i.e., fewer tape-strips were needed to remove a larger fraction of the barrier (Table 3)) confirming that using a fixed number of tape-strips does not guarantee removal of a consistent mass of SC (Tsai et al., 1991; Lademann et al., 2009), and that the amount of SC sampled should be quantified by a suitable method. Nevertheless, it should be pointed out that differential formulation effects on SC collection are less likely to occur when assessing the bioequivalence of a generic product that is Q1 and Q2 equivalent to the reference listed drug.

In the *in vivo* experiment, the 3 products were assessed using duplicate measurements at two time points, resulting in 12 treatment sites per participant, as used in an earlier study comparing 3 econazole creams (N'Dri-Stempfer et al., 2009). The arrangement, though, is flexible such that, if only two products were being compared, for example, additional replicate sites could be included. Replicate measurements are advantageous because they yield values closer to the 'true' population mean, and also provide information about within-subject variability. This is key when deciding, for instance, the appropriate bioequivalence acceptance criteria to use (e.g., 0.8-1.25 versus 0.75-1.33), and/or the appropriate number of participants required to power the study.

The metrics chosen to analyze the IVPT experiments were (a) the cumulative mass of diclofenac that had permeated to the receptor solution after 6 or 8 hours, (b) the cumulative mass of diclofenac that had permeated to the receptor solution after 23 or 24 hours, and (c) the average flux of the drug across the skin either during the period 6-23 hours or 8-24 hours (determined by the difference between the cumulative amounts divided by the time elapsed between those measurements). Statistical inspection of the results revealed exactly the same conclusions as deduced from the *in vivo* experiments: the topical delivery of diclofenac from product P was significantly greater than that from either product S or product V, whereas the permeation of the drug showed no difference from products S and V.

Porcine skin was used in the IVPT experiments for two reasons: first, the limited availability of human skin and, second, because it is considered to be a reliable model for the human barrier (Sekkat & Guy, 2001). However, the results obtained from the three pigs which provided skin for this work demonstrate that the variability seen in human IVPT studies is also duplicated when

porcine skin is used. It follows that, as has become common practice (Franz et al 2009), there is an obvious requirement to carefully match the tissue used in IVPT studies across formulations and to ensure that replicate experiments are performed with skin from multiple donors.

Nonetheless, the fluxes of diclofenac into the viable skin tissue (and beyond) from the 3 products, as determined both *in vivo* and *in vitro*, were in reasonable qualitative *and* quantitative agreement, given both the stark difference between the two types of protocol used (SC tape-stripping *versus* IVPT), and the differences between the subsets of IVPT methods employed. It seems reasonable to suggest, therefore, that *in vivo – in vitro* correlation in topical bioavailability assessment is an achievable goal, and that the judicious selection of complementary tools for measuring the rate and extent of skin uptake, permeation and clearance offers a viable, surrogate strategy to characterize and optimize formulation performance.

5 Conclusion

SC tape-stripping *in vivo* in man and *in vitro* skin permeation experiments using porcine skin have been used to assess the topical bioavailability of diclofenac following application of the drug in three approved and commercialized products. The results from both approaches clearly differentiate one formulation, which delivers significantly more of the active pharmaceutical ingredient, and also reveal that the other two formulations are similar in their performance. There is qualitative and (reasonable) quantitative agreement between the *in vivo* and *in vitro* methods and this correlation provides evidence that the SC tape-stripping technique can also be used to generate information pertinent to the bioavailability of topical drugs whose site of action lies below the skin barrier.

Acknowledgements

We thank Professor Audra Stinchcomb (University of Maryland), Dr. Thomas Franz and Drs. Sam Raney and Priyanka Ghosh (U.S. F.D.A.) for their valuable input into the study. This research was supported by the U.S. Department of Health & Human Services, Food & Drug Administration (1-U01-FD-004947). The views expressed in this paper do not reflect the official policies of the U.S. Food & Drug Administration or the U.S. Department of Health & Human Services; nor does any mention of trade names, commercial practices, or organization imply endorsement by the United States Government

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