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In-vitro permeation of drugs into porcine hair follicles: is it quantitatively equivalent to permeation into human hair follicles?

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Abstract

It is already well-established that the general permeability properties of porcine skin are close to those of human skin. However, very little is known with respect to drug absorption into hair follicles and the similarities if any between the two types of tissue. The aim of this study was to use the skin sandwich system to quantify follicular drug absorption into porcine hair follicles. To our knowledge, this is the first time that the skin sandwich has been extended to porcine tissue. For this purpose, seven different drugs — estradiol, corticosterone, hydrocortisone, aldosterone, cimetidine, deoxyadenosine and adenosine — exhibiting a wide range of log octanol–water partition coefficients ($\log K_{o/w}$), but comparable molecular weights, were chosen as candidate solutes. The results showed a parabolic profile with maximal follicular contribution occurring at intermediate $\log K_{o/w}$ values. Linear regression analysis indicated that the follicular contributions in porcine skin correlated well with previously published follicular contributions in human skin ($r^2 = 0.87$). The novelty of this research is that we show that porcine tissue is a good surrogate for modelling human skin permeability within the specific context of quantifying drug absorption into hair follicles.

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Introduction

Historically, researchers investigating transdermal drug delivery have debated the relative importance of absorption through the continuous stratum corneum versus penetration via the hair follicles. Early investigators believed that since the follicular orifices occupied merely ~0.1% of the total skin surface area, follicular transport constitutes only a very minor component of total absorption (Scheuplein, 1967; Scheuplein et al 1969). However, more recent studies have implicated the follicles as playing a greater role than previously assumed (Meidan et al 1998; Ogiso et al 2002; Dokka et al 2005; Jung et al 2006; Teichmann et al 2006; Lademann et al 2007; Otberg et al 2007). This development has been associated with the realisation that the hair follicle actually represents an invagination of the epidermis extending down into the dermis, thus providing a greater actual surface for potential absorption (Meidan et al 2005).

Until recently, a major weakness in this area has been that quantification of follicular transport has remained elusive due to a lack of appropriate methodologies. The use of animal skin remains problematic as it is always difficult to ascertain that the barrier properties of any follicle-containing membrane are identical to those of the control follicle-free membrane. Imaging systems may yield a semi-quantitative static view of what is really a highly dynamic process, although ongoing technological advances mean that this approach is continually improving (Grams et al 2005; Jacobi et al 2005; Stracke et al 2006). In the last few years, a range of promising follicular orifice-blocking techniques have been devised (Jung et al 2006; Teichmann et al 2006; Lademann et al 2007; Otberg et al 2007). These have generated considerable interest, although the methodologies require some specialist expertise to effect.

One simple quantitative in-vitro technique that has emerged in recent years is the skin sandwich system (Barry 2002, Essa et al 2002). Essentially, the role of the shunts in total percutaneous absorption is determined by comparing drug flux across hydrated epidermal membrane with that through a hydrated sandwich of epidermal membrane plus adhering extra stratum corneum on top. As shown in Figure 1, the extra stratum corneum blocks all available shunts such as follicles. If the shunts route plays no part in the permeation process then steady-state flux through the sandwich is half that of the single membrane. Conversely, if shunts facilitate all drug transport then flux through the sandwich is zero. Thus, the magnitude of flux decrease allows quantification of the shunt contribution to total absorption. The shunts are believed to be the hair follicles, since the much smaller sweat-duct openings shut in highly hydrated tissue. Comprehensive mathematical descriptions and validations of the skin sandwich system have been published (Barry 2002; Essa et al 2002).

Although it is known that the permeability properties of porcine skin are fairly similar to those of human skin, very little is known about the tissue's equivalency in terms of follicular drug absorption. Conceivably, it is possible that the two skin species may vary considerably in terms of the follicular component of drug absorption. The aim of the current study is to shed light on this issue. To this end, we applied the skin sandwich system to porcine skin – the first time that this has been done to our knowledge. Seven different solutes of comparable molecular weights (range, 251–362 Da) but exhibiting different log octanol–water partition coefficient ($\log K_{o/w}$) values were tested as candidate molecules. The candidates (with $\log K_{o/w}$ values) were: estradiol (2.29), corticosterone (1.94), hydrocortisone (1.60), aldosterone (1.08), cimetidine (0.40), deoxyadenosine (−0.55) and adenosine (−1.05). The derived follicular component data was compared with follicular component data previously obtained from similar human skin sandwich studies (Frum et al 2007). Linear regression analysis was used to quantitatively compare intrafollicular drug delivery between the two species.

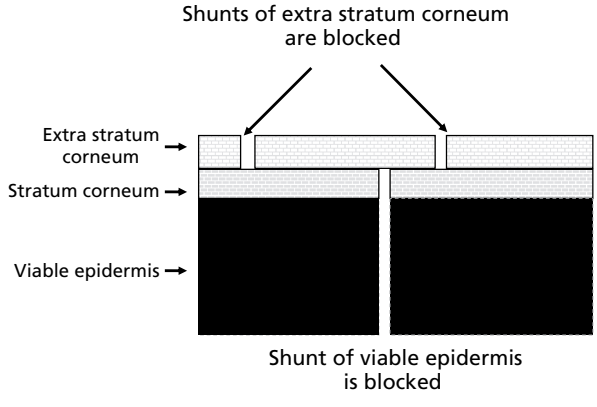

Figure 1 Schematic diagram illustrating the basis of the skin sandwich system.

Materials and Methods

Chemicals

Estradiol, corticosterone, hydrocortisone, aldosterone, cimetidine, adenosine, sodium hydrogen carbonate, bovine pancreatic trypsin (T-4665) and phosphate-buffered saline (PBS) tablets (pH 7.4) were purchased from Sigma-Aldrich (Poole, UK). Tritiated [2,4,6,7-^{3}H]-estradiol (93 Ci mmol^{-1}), [1,2,6,7-^{3}H]-corticosterone (65 Ci mmol^{-1}), [1,2,6,7-^{3}H]-hydrocortisone (74 Ci mmol^{-1}), [1,2-^{3}H]-aldosterone (39 Ci mmol^{-1}), [*N*-methyl-^{3}H]-cimetidine (25 Ci mmol^{-1}) and [2-^{3}H]-adenosine (15 Ci mmol^{-1}) were bought from Amersham Biosciences (Buckinghamshire, UK). Both cold (unlabelled) deoxyadenosine and [8-^{3}H]-deoxyadenosine (10.4 Ci mmol^{-1}) were purchased from MP Biomedicals (Stretton, UK). Sodium azide was bought from Acros Organics (Geel, Belgium). Scintillation fluid (Optiphase HiSafe 3) and vials were purchased from Fisher Scientific (Loughborough, UK) and Packard Instrument Co. (Meriden, CT), respectively. Distilled water was used throughout.

Preparation of epidermal and sandwich membranes

Full-thickness abdominal skin samples were derived from Landrace pigs (a non-pigmented species) that had been slaughtered in a local abattoir. The skins were harvested immediately after the animals' slaughter but before steam sterilisation of the tissue. The skins were cleaned under cold running water and subsequently stored at −80°C for a period not exceeding 6 months. Before each permeation study, the skins were thawed at room temperature and epidermal membranes were prepared by employing the conventional heat separation technique (Kligman & Christophers 1963). To this end, excess fat and adipose tissue were removed and skin sections were immersed for 45 s in a water bath maintained at 60°C. The epidermis was then gently teased off the underlying dermis and floated on a 0.002% w/v sodium azide solution. Stratum corneum (SC) membranes were prepared as described previously (Frum et al 2007). Essentially, epidermal membranes were floated inner side down on an aqueous solution containing 0.0001% trypsin and 0.5% w/v sodium hydrogen carbonate maintained at 37°C. After 12 h, the membranes were picked up on filter paper and remaining digested cells were washed off with water. The SC membranes were then floated on water for 2 h. To hydrate both the epidermal and SC membranes, these were floated with the SC side uppermost on 0.002% sodium azide solution for at least 24 h. Subsequently, SC/epidermal sandwiches were obtained by placing SC membranes on top of epidermal membranes derived from adjacent skin regions.

Further hydration of skin membranes

Franz cells (PermeGear, Bethlehem, PA), exhibiting a diffusion area of 0.64 cm^2 and a receiver cell volume of 5.3 mL, were used to fully hydrate the prepared skin membranes. For this purpose, an aqueous solution containing 0.002% w/v sodium azide was degassed by sonication (Camlab Transsonic T310, Cambridge, UK) for 15 min. The receiver cells were

then filled with this solution while epidermal or SC/epidermal sandwiches were inserted as barrier membranes with the SC side uppermost. The receiver solution was stirred at 600 rev min^{-1} and maintained at 37 ± 0.5°C by a thermostatically controlled water pump (Haake DC10, Karlsruhe, Germany). The donor cells were filled with the same preservative solution and subsequently sealed with Parafilm. The Franz cells were left for 24 h to allow virtually full hydration of the mounted membranes. Subsequently, the sodium azide solution was removed from the donor cell so that permeation studies could be initiated.

Preparation of donor solutions

Each of the test drugs (estradiol, corticosterone, hydrocortisone, aldosterone, deoxyadenosine and adenosine) was prepared as a saturated solution in PBS (pH 7.4), using previously determined saturation solubility values (Frum et al 2007). For cimetidine, it was necessary to use PBS adjusted to pH 8.8. This was undertaken to maintain cimetidine in a totally undissociated form, thus replicating the experimental conditions that were used in our previous human skin study (Frum et al 2007). Crucially, epidermal barrier properties should remain unperturbed at this pH (Sznitowska et al 2001). In all cases, each saturated solution of cold drug was spiked with an appropriate small volume of radiolabelled drug as described previously (Frum et al 2007).

Permeation studies

Permeation studies were initiated by depositing 200 μL of saturated test drug solution on to either hydrated epidermal or hydrated sandwich membranes mounted in Franz cells. The receiver solution was an aqueous solution containing 0.002% w/v sodium azide. At selected time points, a 100-μL volume of receiver phase was withdrawn from each receiver cell and replaced with an equal volume of blank receiver phase. Each 100-μL sample was subsequently vortexed with 3 mL of scintillation fluid. Penetrant amounts in the withdrawn samples were determined by liquid scintillation counting (Packard, TriCarb 1600TR). Measured activity values were converted into concentration values and these were then corrected for progressive dilution as described previously (Khan et al 2005).

Each permeation study consisted of 3–5 experimental replicates. For each permeation experiment, a steady-state segment was selected and characterized by linear regression analysis. This analysis was accomplished by using dedicated Microsoft Excel software on an IBM-compatible computer. The percentage follicular contribution was determined according to the relationship:

$$\%\ \text{Follicular contribution} = [1 - (2 \times J_{Sand}/J_{Ep})] \times 100 \quad (1)$$

where J_{Sand} and J_{Ep} are the steady-state flux values for the sandwich and single epidermis, respectively.

Statistical analysis

For each test drug, the difference between its steady-state flux through the single epidermal membrane and its steady-state flux through the sandwich membrane was analysed by applying the Mann–Whitney U-test (Jones 2002). $P < 0.05$ denoted significance. Each flux value consisted of 3–5 replicate runs.

Results

Figures 2–5 present graphs of cumulative drug penetration as a function of time for all of the candidate drugs. Each graph is comprised of at least two plots representing single epidermis experiments as well as SC/epidermal sandwich experiments. Table 1 lists all the relevant derived parameters — flux values, kp values, J_{Sand}/J_{Ep} and L_{Sand}/L_{Ep} ratios. Each test solute's saturation solubility in PBS was obtained from a previously published study (Frum et al 2007). At the end of each epidermal membrane study, mean drug concentrations in the receiver cells (to the nearest whole number) were 24, 566, 417, 443, 7341, 4734 and 3775 ng mL^{-1} for estradiol, corticosterone, hydrocortisone, aldosterone, cimetidine, deoxyadenosine and adenosine, respectively. For the sandwich studies, receiver-cell concentrations were obviously lower. Hence, sink conditions were sustained throughout and drug concentrations in the receiver solutions never surpassed 10% of maximum solubility. It is also noteworthy that statistical analysis indicated that for each drug, flux through the sandwich was significantly lower than flux through the single epidermal membrane.

Figure 2 shows the results obtained from the estradiol experiments. It is notable that permeation data were only plotted for 5 and 7 h for single epidermal and sandwich membrane, respectively. This is because the low saturation solubility of estradiol combined with its rapid flux meant that sink conditions would likely not be maintained beyond these time points. Estradiol permeation across single epidermal membranes was characterized by an extremely brief lag phase followed by a mean steady-state flux of 42.12 ng cm^{-2} h^{-1}. This is correlated with a mean kp of 117.10×10^{-4} cm h^{-1}. It is difficult to validate this value by

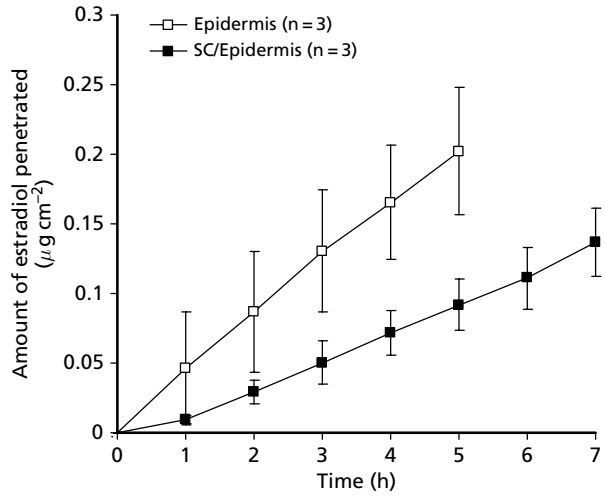

Figure 2 Penetration of estradiol through porcine epidermis and through SC/epidermal sandwich. Error bars represent s.d. values.

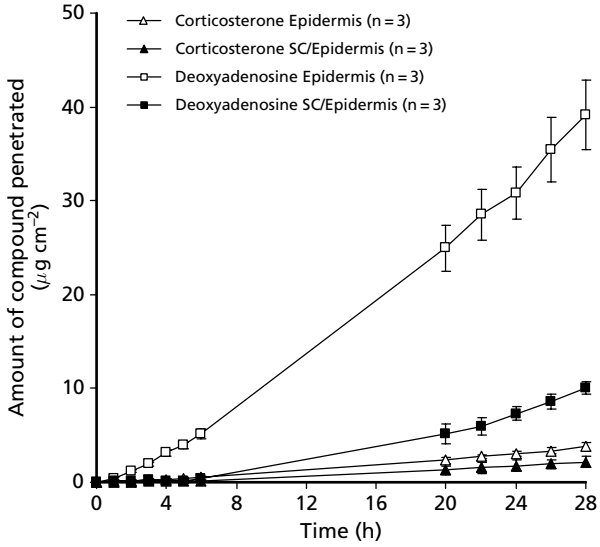

Figure 3 Penetration of corticosterone and deoxyadenosine through porcine epidermis and through SC/epidermal sandwich. Error bars represent s.d. values.

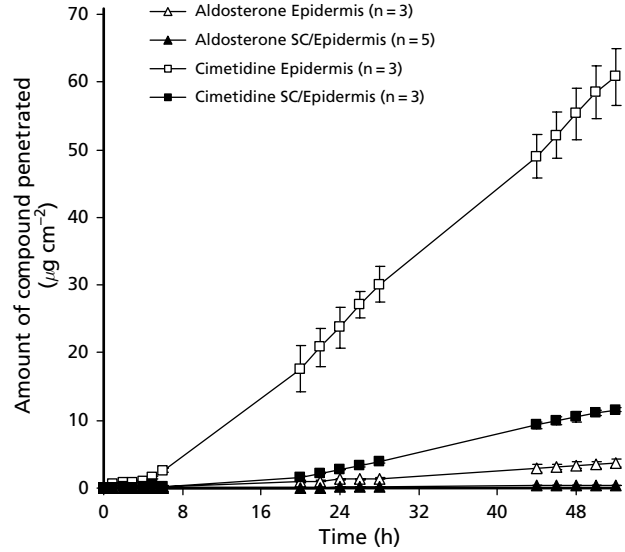

Figure 5 Penetration of aldosterone and cimetidine through porcine epidermis and through SC/epidermal sandwich. Error bars represent s.d. values.

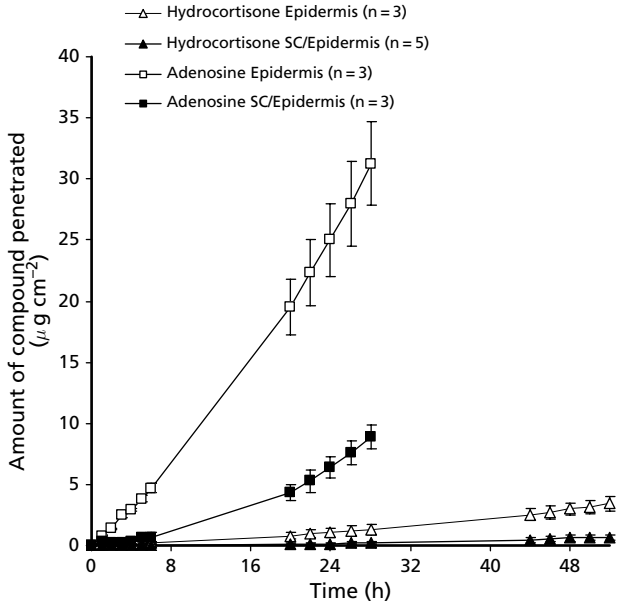

Figure 4 Penetration of hydrocortisone and adenosine through porcine epidermis and through SC/epidermal sandwich. Error bars represent s.d. values.

comparing it with that of other since relatively few literature reports use porcine epidermis as a barrier. However, porcine and human epidermal membrane permeabilities are typically the same order of magnitude (Schmook et al 2001). Within this context, our measured kp value is between 1.5- and 4-fold greater than previous in-vitro human epidermal studies, where reported mean kp values were 61.2×10^{-4} cm h^{-1} (Knuston et al 1993), 30×10^{-4} cm h^{-1} (Mitragotri 2001), 67.0×10^{-4} cm h^{-1} (Essa et al 2002) and 83.2×10^{-4} cm h^{-1} (Suhonen et al 2003). Passive penetration of estradiol across the SC/epidermal sandwich was characterised by a lag time that was 3.17 times longer. This is not far from the theoretical result where doubling membrane thickness should cause a quadrupling of the lag time of a diffusing solute (Barry 2002). However, greater accuracy can probably be achieved by comparing the steady-state flux values for the single membrane and sandwich. As Table 1 indicates, the measured value of J_{Sand}/J_{Ep} was 0.46. By applying equation 1, this yields a follicular contribution of 8%.

With respect to corticosterone (Figure 3), permeation across single epidermal samples was characterised by a brief lag time followed by a mean steady-state flux of 205.36 ng cm^{-2} h^{-1}. By taking into account the concentration of corticosterone in the donor compartment, we were able to calculate a mean kp value of 14.33×10^{-4} cm h^{-1}. Again, no porcine epidermal kp values could be found in the literature reports. Yet our kp value is about 2- to 4-fold higher than values documented in the literature for human epidermal flux of corticosterone. For example, Buchwald & Bodor (2001) and Suhonen et al (2003) reported respective mean kp values of 3.2×10^{-4} cm h^{-1} and 5.8×10^{-4} cm h^{-1}. Regarding the sandwich membrane studies, the lag phases were much longer (5.07 times), as might be expected. The calculated J_{Sand}/J_{Ep} value was 0.54. Mathematically, this gives a follicular contribution of −8%, which is obviously illogical. This illustrates that the sandwich system yields errors arising from the inherent variability in skin harvested from different sites (Essa et al 2002). The value of 0.54 can be rounded down to 0.50, indicating that follicular shunts play no role in corticosterone penetration.

For the hydrocortisone studies (Figure 4), permeation was allowed to proceed for 52 h, since the lag times were appreciably longer than for estradiol or corticosterone. During the

Table 1 Solubility and epidermal permeation properties of the candidate solutes

Candidate Solute	Concn PBS (mg mL^{-1})[a]	J_{Ep}[b] (ng cm^{-2} h^{-1})	Epidermal kp (cm h^{-1} $\times 10^{-4}$)	J_{Sand}[b] (ng cm^{-2} h^{-1})	J_{Sand}/J_{Ep}	Lag $time_{Ep}$ (h)	Lag $time_{Sand}$ (h)	L_{Sand}/L_{Ep}
Estradiol	0.0036 ± 0.0000	42.12 ± 1.98	117.10 ± 5.50	19.58 ± 3.35	0.46	0.20 ± 0.04	0.64 ± 0.20	3.17
Corticosterone	0.12 ± 0	205.36 ± 59.27	17.41 ± 4.94	110.50 ± 1.86	0.54	1.73 ± 0.16	8.78 ± 4.31	5.07
Hydrocortisone	0.29 ± 0.03	59.80 ± 20.13	2.03 ± 0.69	20.40 ± 3.74	0.34	9.65 ± 1.86	31.11 ± 7.13	3.22
Aldosterone	0.47 ± 0.02	86.97 ± 15.68	1.83 ± 0.33	15.61 ± 1.88	0.18	8.03 ± 1.60	22.53 ± 3.14	2.80
Cimetidine	5.46 ± 0.23	1527.37 ± 164.09	2.79 ± 0.30	318.45 ± 53.61	0.21	4.47 ± 0.69	15.46 ± 0.33	3.46
Deoxyadenosine	6.38 ± 0.47	1724.53 ± 200.34	2.70 ± 0.31	624.35 ± 79.65	0.36	3.10 ± 0.05	11.83 ± 3.36	3.82
Adenosine	6.04 ± 0.11	1461.94 ± 206.11	2.42 ± 0.34	588.38 ± 5.31	0.40	2.70 ± 0.35	10.22 ± 2.07	3.79

Data are presented as mean ± s.d. [a]Saturation solubilities in PBS at pH 7.4 (or pH 8.8 for cimetidine) were obtained from the literature (Johnson et al 1995; Essa et al 2002; Frum et al 2007). [b]For all candidiate solutes, J_{Sand} values were significantly lower than J_{Ep} values.

steady-state phase, mean hydrocortisone flux across human epidermis was 59.80 ± 20.13 ng cm^{-2} h^{-1}, yielding a mean kp value of 2.03×10^{-4} cm h^{-1}. This value is some 70% higher than a previously published human epidermal permeability measurement of 1.2×10^{-4} cm h^{-1} (Hadgraft & Ridout 1987), yet it almost matches another mean value of 2.3×10^{-4} cm h^{-1} (Johnson et al 1995). Interestingly, mean steady-state hydrocortisone flux across the sandwich reduced to 34% of its value when using the single membrane. Hence, it can be calculated that the follicular contribution was 32%.

Figure 5 shows that aldosterone permeation was associated with a relatively long lag time. Consequently, these experiments were undertaken for a period of 52 h. When using the single epidermal membrane, a mean steady-state flux of 86.97 ng cm^{-2} h^{-1} was measured, corresponding to a mean kp of 1.83×10^{-4} cm h^{-1}. This kp value is 2–6 times higher than the values obtained for human epidermal permeability – 0.58×10^{-4} cm h^{-1} (Johnson et al 1995), 0.3×10^{-4} cm h^{-1} (Mitragotri et al 2000) and 0.97×10^{-4} cm h^{-1} (Suhonen et al 2003). In the sandwich experiments, the mean linear aldosterone flux was only 0.18 of that observed for the single membranes. This means the follicular contribution was 64%.

The single membrane and sandwich membrane penetration profiles for cimetidine are also presented in Figure 5. This compound's permeation through single epidermal tissue was characterised by a moderate lag time followed by a mean steady-state flux of 1527.47 ng cm^{-2} h^{-1}. This is equivalent to an average kp value of 2.79 cm h^{-1}. Unfortunately, we could not find any values in the literature reports with which to compare this value. Steady-state cimetidine flux across the sandwich membranes was only 0.21 of that observed for the single membrane. This means the follicular contribution for cimetidine was 48%.

Figure 3 also shows the penetration–time graphs obtained for the deoxyadenosine studies. Analysis of this data revealed that mean steady state-flux of this drug was 1724.53 ng cm^{-2} h^{-1} for single membrane flux and 624.35 ng cm^{-2} h^{-1} for sandwich membrane flux. This yielded a J_{Sand}/J_{Ep} value of 0.36. Application of equation 1 means that the follicular contribution for deoxyadenosine was 28%.

Determinations of adenosine flux through epidermal membranes yielded a mean steady-state flux of approximately 1462 ng cm^{-2} h^{-1}. Steady-state adenosine flux through SC/epidermal sandwiches was approximately 588 ng cm^{-2} h^{-1} (Figure 4). Hence, the ratio of sandwich flux to epidermal flux was 0.40. This indicates that the follicular contribution for adenosine was 20%.

For each test drug, the percentage follicular contribution was plotted as a function of Log $K_{o/w}$. Figure 6 presents the resulting graph and it can be seen that there is an apparent parabolic relationship. The parabolic curve represents a polynomial equation that fits the data reasonably well ($r^2 = 0.779$). The follicular contribution to flux was small (8% and 0%) for the two most lipophilic solutes, which were estradiol and corticosterone, respectively. With increasing hydrophilicity, the flux contribution increased to 32% for hydrocortisone and 64% for aldosterone. However, further reductions in Log $K_{o/w}$ yielded a trend of progressively

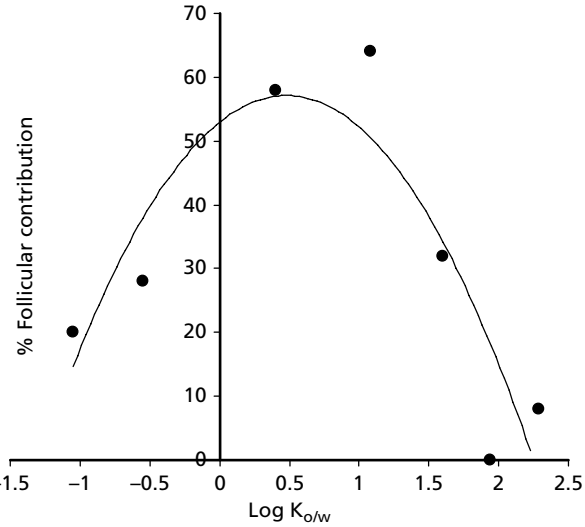

Figure 6 A graph showing the hair follicles' contribution to total flux as a function of solute partition coefficient.

decreasing follicular contributions. The values were 58%, 28 and 20% for cimetidine, deoxyadenosine and adenosine, respectively.

Discussion

It should be initially emphasised that the follicular contribution percentages identified in this report are only approximate values. The sandwich system assumes that the use of replicates makes negligibly small any permeability variations between individual stratum corneum samples. This is not always true and without employing very large numbers of replicates, the system is not optimally robust. This is illustrated by the fact that the follicular contribution for corticosterone ($J_{Sand}/J_{Ep} = 0.54$) was calculated at -8%, which is illogical. Hence, the ratio of 0.54 must be rounded down to a value of 0.50. Thus, we can conclude that our J_{Sand}/J_{Ep} values are prone to a potential error of at least ± 0.04. The system's robustness could be enhanced by running the experiment with much higher number of replicates derived from several individuals. This would yield J_{Sand}/J_{Ep} values exhibiting standard deviations. Suitable statistical tests (Jones 2002) could then be applied to determine significance.

Despite the caveat described above, the parabolic relationship uncovered in this study still appears quite well-defined. Mechanistically, it would be expected that the most lipophilic molecules would preferentially partition into, and diffuse through, the continuous stratum corneum. This is due to the layer's lipoidal nature and the fact that it comprises a much larger fractional area than the follicular orifices. The follicular openings in domestic pig skin typically account for less than $\sim 1\%$ of the skin surface area (Meyer 1986), although it is difficult to be precise due to species and anatomical site-related differences. The most hydrophilic molecules of the seven candidates may also show reduced intrafollicular penetration due to their binding to follicular surfaces. It has been proposed that certain molecules, like mannitol, can hydrogen bond to groups present on the surfaces of follicular pores (Essa et al 2002). Such mechanisms explain the observed parabolic relationship between follicular absorption and hydrophilicity. However, these studies should be run on several dozen more molecules to ascertain that this pattern is indeed representative for most permeants. This is particularly crucial since four out of our current seven candidates were steroids, while adenosine and deoxyadenosine are also structurally-related.

The central aim of this study was to compare the follicular contribution values obtained with porcine skin with those obtained previously for human skin, in which the same solutes were tested (Frum et al 2007). Figure 7 presents a comparative plot of the % follicular contributions for each skin type. Interestingly, linear regression analysis yielded a fairly good correlation coefficient ($r^2 = 0.87$). Of course, it has been known for a long time that porcine skin exhibits permeability properties that are quite similar to those of human skin. Hence, pig skins have been frequently used as an inexpensive, readily available substitute for scarcer human tissue for the purpose of performing drug delivery studies. However,

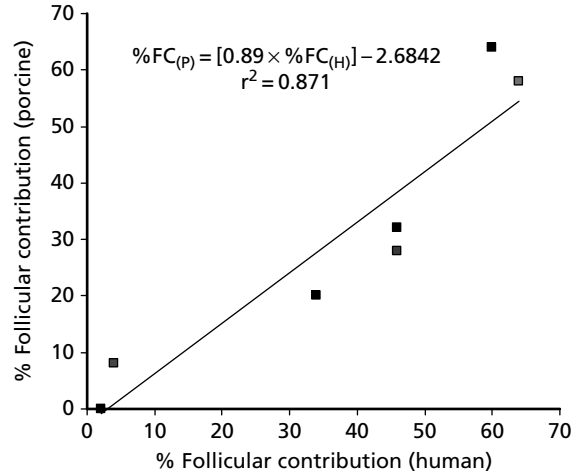

Figure 7 The relationship between follicular contributions in porcine and human skin, as quantified by the skin sandwich system.

less is known about the contribution of hair follicles to percutaneous absorption in this model skin. The results of the current study suggest that for both skin species, the mechanisms modulating selection of follicular penetration are quite similar.

In terms of anatomical site, the porcine skin samples used in this study were harvested from the animals' abdominal region. Although this site is used in drug delivery research (Biruss et al 2007), it is true that porcine ear skin has been more extensively characterised (Meyer et al 2007) and employed as a surrogate barrier (Herkenne et al 2006; Jacobi et al 2007). However, we found in pilot studies that epidermal membranes could be reliably and reproducibly separated out from abdominal tissue while ear samples proved difficult to manipulate.

Although the drug molecules screened in this study were relatively small and uncharged, the skin sandwich system could easily be applied to quantify the follicular permeation of larger or charged molecules. There has recently been considerable interest in drugs that can bind melanin, keratin and other dermal proteins (Banning & Heard 2002; Garnier et al 2007), and the sandwich system would be suitable to test the penetration routes of such agents. However, for compounds that are partially ionised at physiological pH, scintillation counting of radiolabelled compounds would be unsuitable and an analytical method that can distinguish between ionised versus non-ionised species would be required. The skin sandwich could also be employed for testing larger molecules as long as transport was not rate-limited by transfer from the stratum corneum to the underlying hydrophilic layers. As previously discussed (Barry 2002; Meidan et al 2005), the sandwich system cannot be applied in such cases.

Conclusions

This paper reports for the first time that it is indeed possible to employ the skin sandwich experimental system using

porcine tissue. Furthermore, it was found that the absorption of different solutes into porcine hair follicles quantitatively matches the absorption of those solutes into human hair follicles. Hence, the paradigms modulating the extent of intrafollicular drug absorption in the two species are similar. However, further research with either skin type is clearly warranted to fully understand how the penetrant's physicochemical characteristics relate to its penetration route preference.

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