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Report

Use of Shed Snake Skin as a Model Membrane for *in Vitro* Percutaneous Penetration Studies: Comparison with Human Skin

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The potential usefulness of shed snake skin as a model membrane for transdermal research was examined. There are similarities between shed snake skin and human stratum corneum in terms of structure, composition, lipid content, water permeability, etc. The permeability of various compounds and the contribution of several functional groups to the permeability were also found to be similar between shed snake skin and human skin. Moreover, the permeability of compounds through shed snake skin was increased by Azone, one of the most extensively studied transdermal penetration enhancers. Considering the similarities between shed snake skin and human skin, ease of storage and handling, and low cost, shed snake skin may offer a good model membrane for transdermal research.

KEY WORDS: skin penetration; transdermal; shed snake skin; functional group contribution; Azone; *Elaphe obsoleta*.

INTRODUCTION

A variety of model membranes has been used for transdermal research, such as human cadaver skin, hairless mouse skin, and synthetic membranes. Although human skin is the best model membrane, the cost and limited availability put a limitation on the use of human skin. Also, the permeability through human skin varies up to 10-fold depending on the body site (1,39). On the other hand, it is easy to obtain animal skins of the same species with the same line and age. However, the time for experimental use of some animal skins in *in vitro* penetration studies is limited because of deterioration of membrane integrity after prolonged use. Moreover, most animal skins are more permeable than human skin partly because of a larger number of hair follicles (2–5). The use of artificial membranes in transdermal research is limited because they lack keratinized proteins and lipids which are primary components in the stratum corneum of mammalian skins (6–9).

The model membrane examined in the present study is shed snake skin, which is a nonliving pure stratum corneum with no hair follicles (10). Snakes shed their skins periodically, leaving their old stratum corneum behind, which makes it possible to obtain multiple shed skins from the same individual snake. Unlike human stratum corneum, which consists of 10–20 layers of an alpha-keratin-rich intracellular layer and a lipid-rich intercellular layer (11,12), shed snake skin consists of three distinctive layers (Fig. 1) (14,16,37). These are the beta-keratin-rich outermost beta layer, alpha-keratin- and lipid-rich intermediate mesos layer, and alpha-keratin-rich innermost alpha layer (13–14,16). Further, the mesos layer shows three to five layers of multilayer structure with cornified cells surrounded by intercellular lipids, which is similar to human stratum corneum (13,14). This mesos layer is also a major depot of lipids, and the mesos layer and alpha layer are considered to be the main barrier to water penetration through the skin (14–16). Further, water permeability has been compared between shed snake skins from normal snakes and scaleless snakes, which have very thin beta layers, and the water permeability was found to be very similar between normal and scaleless skins (15,17,18). This indicates that the existence of scales may not affect significantly the permeability of compounds through shed snake skin.

Shed snake skins of *Elaphe obsoleta* (black rat snake) have been used in our laboratory, and the similarities between the shed snake skin of this species and the human stratum corneum in terms of thickness and lipid content are summarized in Table I. Lipids are important components of the skin in controlling the permeability of compounds, and delipidization has been shown to increase water permeability through both shed snake skin and human skin (15,19,20). Lipid compositions of shed snake skin and human stratum corneum are also similar, that is, neutral lipids are a major lipid component in both skins and fatty acids, with carbon-chain lengths of C16 and C18 predominant (21–25).

Reported water evaporation rates are also compared between human skin and shed snake skin in Table I. Although

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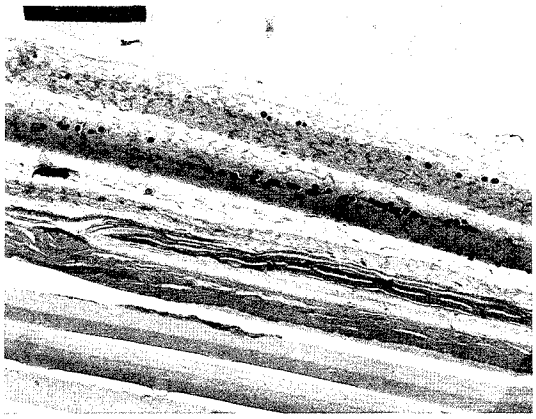

Fig. 1. Electron transmission photomicrograph of a cross section of shed snake skin (*Elaphe obsoleta*). ×3300; reduced 30% for reproduction.

the water permeability through snake skin varies among species and is especially dependent on habitat conditions (15,19), water permeability through shed snake skin of *Elaphe obsoleta* (black rat snake) is very similar to that through human skin (Table II), suggesting that shed skin of this species may offer a good model membrane for transdermal research. In addition, the potential of shed snake skin as a model membrane has been reported using indomethacin with various penetration enhancers (26).

In the present study, permeabilities of several compounds from aqueous solution through shed snake skin were measured and compared with the reported permeabilities through human skin. Also, several functional-group contributions to the permeability of compounds were compared between shed snake skin and human skin. Finally, the effect of Azone on the penetration of compounds through shed snake skin was examined.

Table I. Comparison of Thickness, Lipid Content, and Water Evaporation Rate Between Human Stratum Corneum and Shed Snake Skin

	Human stratum corneum	Shed snake skin (*Elaphe obsoleta*)
Thickness	13–15 μm[a] 10 μm[b]	10–20 μm[c]
Lipid content	2.0–6.5%[d] 3.0–6.8%[e]	ca. 6%[f]
Water evaporation rate	0.1–0.8 mg/cm² hr[g] 0.34 mg/cm² hr[h] ca. 0.2 mg/cm² hr[i]	0.15–0.22 mg/cm² hr[j]

[a] Ref. 12.
[b] Ref. 11.
[c] Ref. 26.
[d] Ref. 22.
[e] Ref. 32.
[f] Ref. 19.
[g] Ref. 33, abdominal skin.
[h] Ref. 1, abdominal skin.
[i] Ref. 20.
[j] Refs. 15 and 19.

Table II. Permeability (×10³ cm/hr) of Various Compounds Through Shed Snake Skin and Human Skin from Aqueous Solutions at 25°C

	Shed snake skin	Human skin
Phenol	5.23 ± 0.36[a]	8.22[b]
m-Cresol	9.88 ± 1.25[a]	15.2[b]
Methylparaben	4.69 ± 0.27[a]	9.12[b]
11α-HPG	0.280 ± 0.017[a]	0.6[c]
Corticosterone	0.102 ± 0.041[d]	0.06[c]

[a] Mean ± SD, n = 3–4.
[b] Ref. 34.
[c] Ref. 35 (at 26°C).
[d] See Table VI.

MATERIALS AND METHODS

Phenol, *p*-hydroxybenzoic acid (p-HBAc), benzoic acid, *m*-cresol, *m*-hydroxyphenylacetic acid (m-HPAA), methylparaben (MP), ethylparaben (EP), propylparaben (PP), butylparaben (BP), 11-alpha-hydroxyprogesterone (11α-HPG), corticosterone, 5-fluorouracil (5-FU), and triamcinolone acetonide (TA) were purchased from Sigma Inc. (St. Louis, Mo.) and used as received. *m*-Hydroxybenzyl alcohol (m-HBA1) was purchased from Aldrich Chemicals (Milwaukee, Wis.) and Azone was supplied by Nelson Research Laboratories (Calif.). All the other chemicals were reagent grade and used as received.

In Vitro Penetration Study

Shed snake skins of *Elaphe obsoleta* (black rat snake) were used as the model membrane for *in vitro* penetration studies. Shed snake skins were stored at −20°C prior to use and the dorsal portion was cut into an appropriate size, hydrated at 40°C for 30 min, and mounted on a diffusion cell.

For the comparison study between shed snake skin and human skin, each compound was dissolved in pH 7.2 isotonic phosphate buffer for a donor solution and the penetration from the solution through shed snake skin was measured at 25°C with an automated diffusion-cell system as previously reported (27). The donor concentration was 1–3 mg/ml for each compound and the UV absorbance of the receptor solution (pH 7.2 isotonic phosphate buffer) was monitored at 5-min intervals at 272, 268, and 255 nm for phenol, *m*-cresol, and methylparaben, respectively. The penetration of corticosterone and 11α-HPG was measured at 25°C with a Franz-type diffusion cell (27) and the sampled receptor solution (pH 7.2 isotonic phosphate buffer) was analyzed with HPLC. Saturated solutions in pH 7.2 isotonic buffer were used as the donor solution.

For the group contribution study, each compound was dissolved in pH 3.0 phosphate buffer (0.01 *m*) as the donor solution, except for the parabens, in order to measure the penetration of the neutral species. Parabens were dissolved

in pH 7.2 isotonic buffer, which was then used as the donor solution. The donor concentration was 3–7 mg/ml for phenol, benzoic acid, p-HBAc, m-cresol, and m-HBA1 but a higher concentration (15 mg/ml) was used for m-HPAA. The donor concentrations for parabens were 0.1–0.3 mg/ml. The receptor solution was pH 7.2 isotonic phosphate buffer for all of the compounds and the penetration was measured at 37°C with an automated diffusion cell system. The UV absorbance of the receptor solution was monitored at 5-min intervals at 272 nm for *m*-cresol, m-HBA1, and m-HPAA and at 254, 268, 270, and 255 nm for p-HBAc, phenol, benzoic acid, and the parabens, respectively.

For the enhancer study, pure Azone was applied on the shed snake skin 12 hr prior to the experiment and the penetration of 5-FU and TA was measured and compared with those through untreated shed snake skin. A saturated solution in pH 7.2 isotonic buffer was used as the donor solution for each compound and the penetration was measured at 37°C with pH 7.2 isotonic buffer as the receptor solution. A Franz-type diffusion cell was used and the sampled receptor solution was analyzed with HPLC.

For the study to examine the variability among shed snake skins, the penetration of methylparaben and corticosterone was measured from aqueous solution through shed skins from several different snakes of the same species (*Elaphe obsoleta*). Methylparaben penetration was measured at 37°C with an automated diffusion-cell system and the UV absorbance of the receptor solution was monitored at 255 nm. The donor solution contained 0.1–1.0 mg/ml drug in pH 7.2 isotonic buffer. Corticosterone penetration was measured at 25°C with a Franz-type diffusion cell and the sampled receptor solution was analyzed with HPLC. A saturated solution in pH 7.2 isotonic buffer was used as the donor solution. The receptor solution was pH 7.2 isotonic phosphate buffer.

HPLC Conditions

Corticosterone, 11a-HPG, and TA were analyzed using a RP-18 column (Spheri-5, Brownlee Laboratories, Calif.) with a mobile-phase composition of $CH_3CN:H_2O$ = 44:56 for a 11α-HPG and $CH_3CN:H_2O$ = 40:60 for corticosterone and TA. The flow rate was 0.8 ml/min, with UV detection at 241 nm for each compound. 5-FU was analyzed with a μBondapak C18 column (Waters Associates, Milford, Mass.) using 0.05 *M* KH_2PO_4–H_3PO_4 buffer (pH 3.0) as the mobile phase. The flow rate was 1.0 ml/min, with UV detection at 266 nm.

RESULTS AND DISCUSSION

Figure 2 shows the penetration of phenol and *m*-cresol through shed snake skin from an aqueous solution at 25°C. Although the data are shown for every 30 min in Fig. 2, the penetration was monitored at 5-min intervals as mentioned earlier. Penetration was very rapid and almost no lag time was observed for each compound. Methylparaben showed a similar penetration profile and the permeabilities of these compounds were calculated from the initial straight portion of the penetration curve according to Eq. (1), with less than 5% of the drug penetrating through the skin.

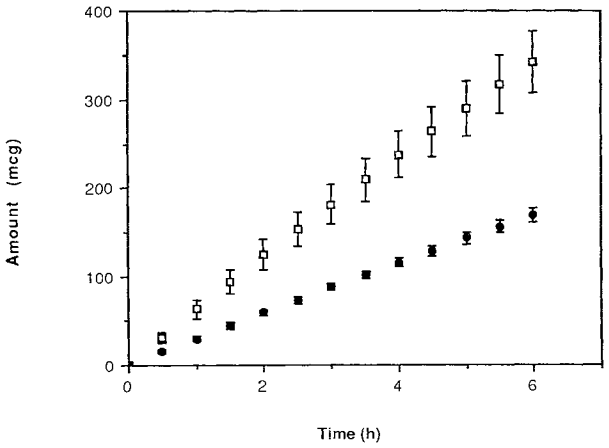

Fig. 2. Penetration of *m*-cresol (□) and phenol (●) from aqueous solutions through shed snake skin (mean ± SE, n = 3–4).

$$P = \frac{dQ}{dt}\frac{1}{A}\frac{1}{C_d} \quad (1)$$

where P = the permeability, dQ/dt = the slope of the straight portion of the penetration curve, A = the surface area (1.8 cm^2 for the diffusion cells used in the present study), and C_d = the drug concentration in donor phase. The permeabilities thus calculated are listed in Table II for each compound.

Penetration profiles of 11α-HPG and corticosterone from an aqueous solution through shed snake skin are shown in Fig. 3. These two compounds showed much slower penetration than the above three compounds and 6- to 8-hr lag times were observed for 11α-HPG and corticosterone. Permeabilities were again calculated from the straight portion of the penetration curve according to Eq. (1) and the results are listed in Table II. Also listed in Table II are the reported permeability values of these compounds from aqueous solution through human skin. As shown in Table II, permeability through shed snake skin is quite similar to that through human skin for each compound. For rat or mouse skins, however, it is not unusual to observe several times larger permeabilities than for human skin (2,4,5,38). In the extreme

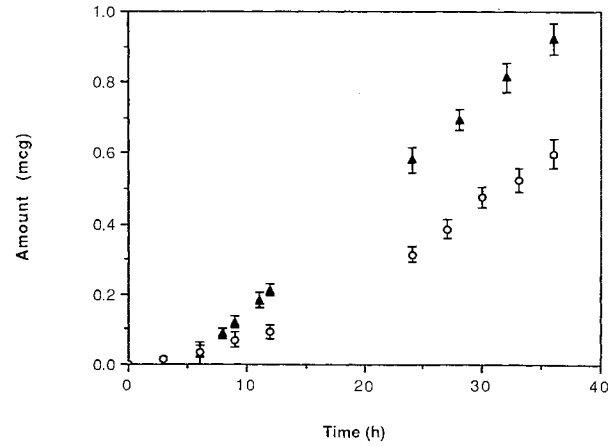

Fig. 3. Penetration of 11α-HPG (▲) and corticosterone (○) from aqueous solutions through shed snake skin (mean ± SE, n = 3–5).

case, the paraquat permeability through hairless mouse skin is more than 1000 times greater than through human skin (4). Also, the permeabilities for human skin listed in Table II are those through abdominal skin, which has been shown to be one of the least permeable sites in the body (1,39). Considering these facts, shed snake skin may offer a good model membrane for transdermal research. Usually shed snake skin penetration studies provide conservative estimates for human skin penetration since it is less permeable than human skin for most compounds.

Permeability values through shed snake skin at 37°C are listed in Table III for parabens, phenol, benzoic acid, p-HBAc, *m*-cresol, m-HBA1, and m-HPAA. The free energy of transfer of the methylene group from the aqueous phase to shed snake skin was calculated from the permeability data for parabens according to the following equation:

$$\Delta(\Delta G) = -RT\,\Delta\ln(P) \qquad (2)$$

where $\Delta(\Delta G)$ = the free energy of transfer of the methylene group from the aqueous phase to shed snake skin, P = the permeability, R = the gas constant, T = the absolute temperature, and $\Delta\ln(P)$ = the slope of $\ln(P)$ vs carbon number plot (carbon numbers 1–4 for methyl-, ethyl-, propyl-, and butylparaben). The free energy of transfer thus calculated for the methylene group is listed in Table IV. The free energies of transfer of the hydroxyl and carboxyl groups were calculated from the permeability data listed in Table III according to the following equation:

$$\Delta(\Delta G)_x = -RT\,ln(P_{RX}/P_{RH}) \qquad (3)$$

where $\Delta(\Delta G)_x$ = the free energy of transfer of the functional group X from the aqueous phase to shed snake skin, R = the gas constant, T = the absolute temperature, P_{RX} = the permeability of the test compound, and P_{RH} = the permeability of the reference compound. The free energy of transfer of the hydroxyl group was calculated from two combinations of the test and reference compounds, that is, RX = p-HBAc and RH = benzoic acid, or RX = m-HBA1 and RH = *m*-cresol. Also, for the carboxyl group, RX = p-HBA and RH = phenol, or RX = p-HPAA and RH = *m*-cresol. The free energy values thus calculated for the hydroxyl and carboxyl groups are listed in Table IV. The negative free energy value for the methylene group and the positive values for the hydroxyl and carboxyl groups indicate that shed snake skin is a lipophilic membrane and that lipophilic functional groups (e.g., methylene group) increase the permeability of compounds through shed snake skin but that hydrophilic groups (e.g., hydroxyl or carboxyl group) decrease the permeability. Also, the functional-group effects on the permeability appear to be similar between the hydroxyl and the carboxyl groups, which is reflected in the similar free energy values for these two functional groups.

Table III. Permeability of Various Compounds Through Shed Snake Skin from Aqueous Solutions at 37°C

	Permeability ($\times 10^3$ cm/hr)[a]
Methylparaben	2.67 ± 0.30
Ethylparaben	4.97 ± 0.96
Propylparaben	8.75 ± 1.02
Butylparaben	22.6 ± 0.48
Phenol	16.2 ± 0.87
Benzoic acid	24.3 ± 3.1
p-HBAc	0.51 ± 0.14
m-Cresol	18.5 ± 0.14
m-HBA1	0.337 ± 0.048
m-HPAA	0.34 ± 0.12

[a] Mean ± SD, n = 3–4.

Table IV. The Free Energy of Transfer of Various Functional Groups from an Aqueous Phase to the Stratum Corneum at 37°C Calculated from the Permeabilities of Several Compounds

Functional group	$\Delta(\Delta G)$ (cal/mol)	
	Shed snake skin[a]	Human skin
$-CH_2-$	−435 ± 17	−440[f]
–OH	2390 ± 190[b]	2400[f]
	2470 ± 110[c]	
–COOH	2220 ± 110[d]	1500[f]
	2500 ± 260[e]	

[a] Mean ± SD, n = 3–4.
[b] RX = p-HBAc, RH = benzoic acid.
[c] RX = m-HBA1, RH = *m*-cresol.
[d] RX = p-HBAc, RH = phenol.
[e] RX = m-HPAA, RH = *m*-cresol.
[f] Ref. 36.

The reported free energy values for the transfer of the methylene, hydroxyl, and carboxyl groups from the aqueous phase to the human skin are also listed in Table IV. The free energies of transfer of these functional groups appear to be similar when one compares shed snake skin with human skin, suggesting similar membrane characteristics between shed snake skin and human skin. This observation again supports the use of shed snake skin as a model membrane for preliminary transdermal research.

Penetration of 5-fluorouracil (5-FU) and triamcinolone acetonide (TA) from aqueous solutions through untreated shed snake skin are compared to that through Azone-treated shed snake skin in Table V. Penetration of both 5-FU and TA was significantly increased by Azone pretreatment but the effect of Azone was much larger on the 5-FU penetration (more than ×200) than on the TA penetration (ca. ×7). It has been reported in hairless mouse or rat skin that 5-FU penetration was increased by about 100 times by Azone (28,29) but the effect of Azone on TA penetration through hairless

Table V. Penetration of Triamcinolone Acetonide (TA) and 5-Fluorouracil (5-FU) Through Untreated and Azone-Treated Shed Snake Skin

	Penetration of TA at 6 hr	
Untreated skin	0.27 ± 0.06 μg[a]	(0.82 ± 0.18%)[b]
Treated skin	2.00 ± 0.23 μg	(6.10 ± 0.70%)
	Penetration of 5-FU at 12 hr	
Untreated skin	48.4 ± 6.1 μg	(0.13 ± 0.017%)
Treated skin	10.6 ± 0.74 mg	(29.5 ± 2.1%)

[a] Percentage of the drug in the donor phase.
[b] Mean ± SE, n = 4–5.

mouse skin appears to be variable, from an 8-fold increase (30) to a more than 100-fold increase (7). In those hairless mouse skin studies, however, an Azone solution or emulsion was used as the donor solution or for pretreatment. Since pure Azone was used in the present study for the pretreatment of shed snake skin, the penetration enhancement effect may have been more pronounced in the present study compared to that in the hairless mouse skin studies. Indeed, penetration enhancement by Azone is less marked in human skin than that in hairless mouse skin (31). Although the magnitude of the 5-FU and TA penetration enhancement by Azone was similar between shed snake skin and hairless mouse skin, further studies will be needed to determine if the shed snake skin is more similar to hairless mouse skin than to human skin in terms of its response to transdermal penetration enhancers.

Finally, the penetration of methylparaben and corticosterone was measured using shed snake skins from four different snakes in order to examine the variability in permeability among different snakes. Penetration of methylparaben and corticosterone from aqueous solution through untreated shed snake skin was measured and the permeabilities were calculated according to Eq. (1). The results are summarized in Table VI. For methylparaben, the greatest difference in permeability was observed between snake skin No. 1 and snake skin No. 2, which showed a difference by a factor of about 1.7. For corticosterone, on the other hand, the greatest difference was observed between snake skin No. 5 and snake skin No. 6, which differed in permeability by a factor of 2.5. Although the permeability was somewhat variable among shed skins from different snakes, the differences were relatively small and may not be a major concern. Also, the relatively small standard deviations for each snake in Table VI indicate little variability from different sites of each shed skin, since the shed snake skin pieces (30–40 pieces obtained from one shed) were selected randomly in this study.

The present study has confirmed that there are similarities between shed snake skin and human skin in terms of structure and composition, the permeability of several compounds, and the contribution of several functional groups to the permeability. Azone, one of the most extensively studied transdermal penetration enhancers, was also shown to increase the penetration of 5-FU and TA through shed snake skin in the same manner as it does in hairless mouse skin.

Table VI. Permeability of Methylparaben and Corticosterone Through Shed Skins from Different Snakes

Snake No.	Methylparaben permeability ($\times 10^3$ cm/hr)[a]	Snake No.	Corticosterone Permeability ($\times 10^5$ cm/hr)[b]
1	4.10 ± 1.70 (n = 4)	5	5.9 ± 1.0 (n = 5)
2	2.67 ± 0.30 (n = 3)	6	15.0 ± 1.3 (n = 6)
3	4.42 ± 0.76 (n = 4)	7	8.9 ± 3.0 (n = 5)
4	3.76 ± 1.00 (n = 3)	8	9.5 ± 0.9 (n = 4)
	3.82 ± 1.20 (n = 14)[c]		10.2 ± 4.1 (n = 20)[c]

[a] Permeability at 37°C, mean ± SD.
[b] Permeability at 25°C, mean ± SD.
[c] Grand mean ± SD.

However, further studies will be needed to clarify the similarity between shed snake skin and human skin in terms of the response to various transdermal penetration enhancers. Overall, shed snake skin may be a useful model membrane for some transdermal research because of its similarities to human skin, ease of storage and handling, and low cost.

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