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Characteristics of Shed Snake Skin Permeability to Indomethacin and Fatty Alcohols

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Abstract

To investigate the utilities of a shed snake skin as a model membrane for preclinical studies of transdermal drug delivery, the flux of indomethacin was determined under various conditions by using a diffusion cell. The flux of fatty alcohols was determined and compared with that in human skin reported in references. The esterase activity of shed snake skin was also determined.

It was found that the flux of indomethacin decreased with an increase of pH and the amount of ethanol in a vehicle. The flux of indomethacin increased by the addition of Azone, *N*-methyl-2-pyrrolidone and *N,N*-dimethyl-*m*-toluamide in the cream.

The flux of fatty alcohols in shed snake skin was greater than that reported in human skin, and shed snake skin had similar esterase activity to human skin.

Skin provides an advantageous route of drug administration over the oral route (Guy & Hadgraft 1987). In particular, transdermal systemic delivery of drugs gives a sustained pharmacological action and the drug delivery can be easily discontinued if adverse reactions occur.

In an in-vitro percutancous absorption study, the use of human skin would be preferred for screening development formulations. However, because of the limitation of the use of human skin, animal skins (Bond & Barry 1988; Hing et al 1989; Harada et al 1993) and artificial membranes have been examined as model membranes for human skin. It has been reported that there are species variation in skin permeability (Tregear 1966; Bartek et al 1972; Wester & Maibach 1975; Shnha et al 1978; Harada et al 1993) as well as a marked regional variation in human skin permeability (Feldmann & Maibach 1967; Rougier et al 1986; Harada et al 1993).

These findings suggest the use of different model membranes for human skin corresponding to different anatomic sites is reasonable.

Recently, the usefulness of shed snake skin as a model membrane has also been reported (Ibuki 1985; Higuchi & Konishi 1986; Nghiem & Higuchi 1988).

In the present study, characteristics of in-vitro shed snake skin permeability to indomethacin and fatty alcohols were studied to investigate the utility of a shed snake skin as a model membrane for preclinical studies of transdermal drug delivery. Indomethacin was selected as a model permeant of organic weak electrolytes because its externally applied preparations have been clinically used and many studies on shed snake skin permeability to indomethacin have been made (Fleeker et al 1989; Hirvonen et al 1991). Aliphatic alcohols were also used as lipophilic and non-dissociated permeants. Furthermore, considering the percutancous application of antedrugs, hydrolytic activity of the skin should be examined. Therefore, esterase activity of the shed snake skin was also examined. To examine the possibility of the use of shed snake skin for preclinical screening of absorption enhancers for percutaneous preparations, the effects of Azone and other absorption enhancers on the permeation of indomethacin were also examined.

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Materials and Methods

Materials

Indomethacin and dimethylsulphoxide (DMSO) were purchased from Wako Chemical Co., Ltd. Di-isopropanolamine, *N*-methyl-2-pyrrolidone, *p*-nitrophenyl acetate and *p*-nitrophenol were purchased from Nacalai Tesque. Polyoxyethylene laurylether was supplied by Nikko Chemicals Ltd. *N,N*-Dimethyl-*m*-toluamide was purchased from Tokyo Chemical Industry. Egg lecithin was supplied by Asahi Chemical Industry Co., Ltd. Azone was supplied by Nelson-Sumisho Co., Ltd. All solvents were analytical grade and were used without further purification. Inteban (1% indomethacin solution, Sumitomo Pharm.Co.), Idomethine Kowagel (1% indomethacin gel) and Idomethacine Kowa Cream (1% indomethacin cream, Kowa) were used for the liquid, gel and cream preparations of indomethacin.

Shed snake skin

Shed snake skin of *Naja naja* Kaoupha or *Pythommolus bivttatus* was provided by Kourankei snake centre, Nagoya, Japan. The shed snake skin was washed with water and allowed to dry at room temperature. They were stored at 32°C, 70% relative humidity. Dorsal skin was used after overnight prehydration in an isotonic pH 7·4 Tris-HCl buffer before use (Ibuki 1985).

Preparation of test solution and incorporation of an enhancer into indomethacin commercial preparations

Indomethacin was dissolved in a definite volume of ethanol, then distilled water was added to obtain a solution of a definite volume ratio of ethanol to water because of limitation of

solubility of indomethacin in water.

Small amounts of di-isopropanolamine or 1 M HCl was added to adjust the pH of the solution (Inagi et al 1981). Final concentration of indomethacin in the dosing solution was 0·5 mg mL^{-1}.

An enhancer was added to the indomethacin-gel and the indomethacin-cream and mixed using a pestle and mortar. The appearances of the gel or cream was not changed by addition of any of the enhancers, apart from Azone, where transparent indomethacin gel became translucent, possibly because the Azone was not solubilized. Enhancers were also dissolved in indomethacin solutions. Experimental and control groups were compared, using Student's *t*-test for paired observations.

In-vitro permeation studies

A side-by-side diffusion cell (Higuchi & Konishi 1986) was used. A circular piece of shed snake skin was held securely between the two halves of the cell. The area of the skin exposed to the test solution was 1·77 cm^2 (0·75 cm diam.). A gel preparation (0·5 g), a cream preparation (0·5 g) or a test solution (6 mL) was placed in the donor cell. Isotonic tris-HCl buffer (pH 7·4) was filled into the receptor cell (19 mL). The apparatus was kept at 32 ± 0·5°C in a water bath. Portions of the solution (0·6 mL) in the receiver cell were taken out at designated time intervals for 24 h. The same volume of the buffer solution was resupplied in the receiver cell after each sampling.

Measurement of esterase activity

Extraction of esterase from shed snake skin. Enzymes in the shed snake skin were extracted by immersing skin in 0·1 M phosphate buffer (pH 7·2) at 32°C for 24 h (Kobayashi 1964).

The extracted solution was centrifuged at 3000 rev min^{-1}, and the supernatant was used as an enzyme solution.

Rate of hydrolysis. One millilitre of acetone containing various concentrations of *p*-nitrophenyl acetate (0·3–1·0%) was mixed with 0·1 M phosphate buffer (pH 7·2) to give a final volume of 100 mL. Four millilitres of the solution was kept at 32°C, then 0·2 mL enzyme solution was added and incubated at 32°C for 6 h. The concentration of the hydrolysed *p*-nitrophenol was measured spectrophotometrically at 400 nm Kobayashi 1964; Nghiem & Higuchi 1988). The non-enzymatic hydrolysis was also performed at 32°C. The enzymatically hydrolysed amount of *p*-nitophenol was obtained by subtracting the values in the non-enzymatic hydrolysis from that after incubation with enzyme solution.

Analysis

Indomethacin. The concentration of indomethacin was determined spectrophotometrically at 265 nm with a Shimazu spectrophotometer, model 160.

Aliphatic alcohols. The concentration of aliphatic alcohols was gas-chromatographically analysed using a Perkin Elmer Gas Chromatograph. The glass column, 1·8 m × 2·0 mm, packed with 25% PEG 20M 60/80 mesh (Nishiokogyo Co. Ltd.) was used.

Results

Penetration of indomethacin through shed snake skin

The flux of indomethacin was calculated from the slope of a regression line obtained by plotting the penetrated amount through shed snake skin from 50% (v/v) ethanolic aqueous solution having different pH against time. As shown in Fig. 1, the flux of indomethacin was decreased with an increase of pH of 50% ethanolic aqueous solution, corresponding to the pH-partition theory except at pH 7·0. The flux of indomethacin at pH 7·0 was greater than that expected from pH-partition theory. Similar results are reported in the penetration of indomethacin through guinea-pig skin (Inagi et al 1981). Unexpected results in the present study at pH 7·0 may be due to possible ion-pair formations between indomethacin and di-isopropanolamine, which was used for adjusting pH of the test solution.

Effect of ethanol in the vehicle on the penetration of indomethacin

To examine the effect of ethanol in the vehicle, the flux of indomethacin through shed snake skin was determined in the range from 30 to 100% (v/v) ethanol. As shown in Fig. 2, the flux of indomethacin decreased with an increase of the amount of ethanol. The following equation has been proposed to describe the process of a drug penetration under steady state conditions:

$$J = D_m(C_v/S)/(L\gamma) \quad (1)$$

where J is the flux of a drug through shed snake skin, D_m is the diffusion coefficient of the drug through shed snake skin, C_v is the concentration of the drug in a vehicle, S is the solubility of the drug in a vehicle, γ is the effective activity coefficient of the drug in the skin barrier, L is the effective barrier thickness, and (C_v/S) is the so-called thermodynamic activity of the drug in the vehicle.

As shown in Fig. 3, a linear relationship between the flux and (C_v/S) is observed. This result means that the flux of indomethacin is closely related to the thermodynamic activity of indomethacin in the vehicle. Therefore, the decrease in the flux of indomethacin as the concentration of ethanol in the

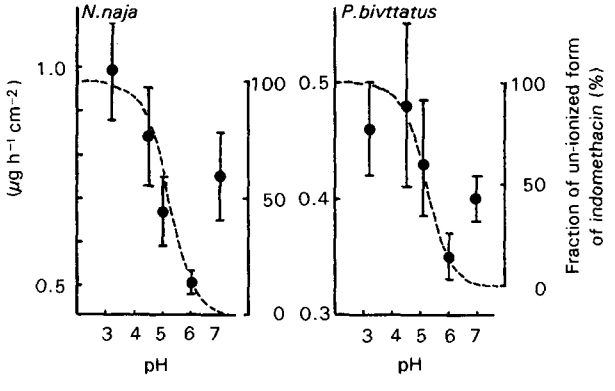

FIG. 1. Effect of pH on flux of indomethacin from 50% ethanolic aqueous solution through *N. naja* and *P. bivttatus* shed snake skin. Results are represented as mean ± s.e.m. (n = 3). The dotted curve represents the theoretical value of the undissociated fraction of indomethacin.

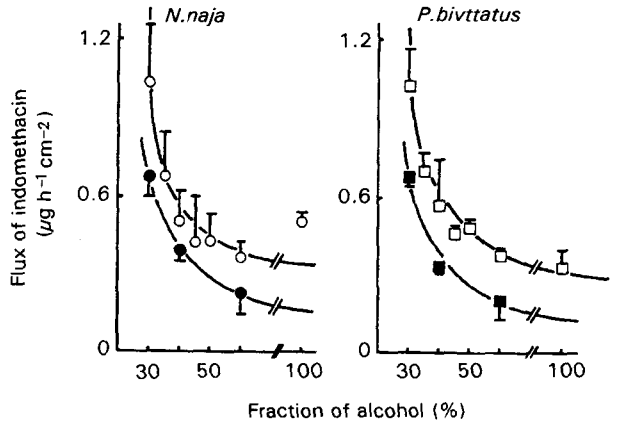

FIG. 2. Relationship between flux of indomethacin and fraction of alcohol in water. Results are represented as mean ± s.e.m. (n = 3). Ethanol ○, □; isopropanol ●, ■.

vehicle increased will be subjected to the decreased thermodynamic activity of indomethacin in the vehicle.

Effect of the penetration enhancer on indomethacin penetration from gel or cream preparation

Enhancers such as surfactants, aprotic solvents and Azone have been investigated to evaluate their enhancing effect. Azone and primary alcohols are reported to increase the flux of indomethacin (Tsuzuki et al 1988). In the present study, effects of enhancers on the flux of indomethacin through shed snake skin was determined by using commercial preparations. From the penetration profiles in the three preparations (1% indomethacin gel, 1% indomethacin cream and 1% indomethacin liquid solution) (Fig. 4), the amount of indomethacin which penetrated through shed snake skin after 24 h was 30·45 (*N. naja*), 33·87 (*P. bivttatus*) μg cm^{-2} in the gel, 8·85 (*N. naja*), 8·66 (*P. bivttatus*) μg cm^{-2} in the cream and 20·96 (*N. naja*), 26·12 (*P. bivttatus*) μg cm^{-2} in the liquid. The difference in the penetrated amounts of indomethacin from the three preparations may be due to the differences in the existing state of indomethacin in the preparations, e.g. dissolved or suspended, or the difference of other constituents in the dosage form.

The influence of six penetration enhancers (Azone, DMSO, *N*-methyl-2-pyrrolidone, polyoxyethylene laurylether, egg lecithin, *N,N*-dimethyl-*m*-toluamide) on indomethacin penetration was investigated in the gel and the cream. As shown in Table 1, the presence of an enhancer in the gel or cream preparations resulted in a significant increase in the flux of indomethacin.

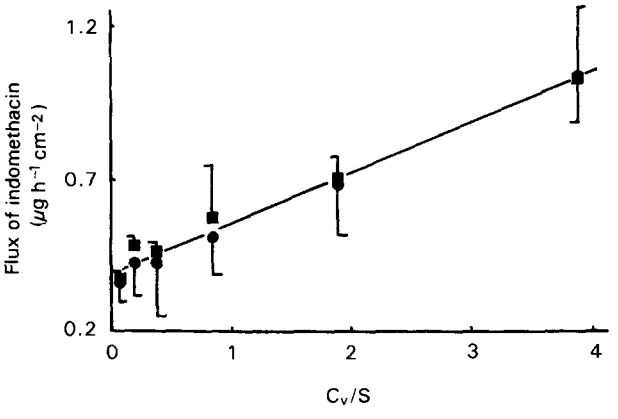

FIG. 3. Relationship between flux of indomethacin and C_v/S. Results are represented as mean ± s.e.m. (n = 3). *N. naja* ●; *P. bivttatus* ■.

Permeability of shed snake skin to aliphatic alcohols

As shown in Table 2, the flux and the permeability constant of the primary alcohols were decreased when the carbon number of the alcohols increased. Relatively hydrophilic alcohols penetrated easily through the shed snake skin. But, it was found that the solubility of the alcohol in the receptor solution influenced its penetration rate. Comparison of the flux of straight chain propanol and butanol and the corresponding branched alcohols resulted in the straight chain alcohols having a higher value of flux, possibly because of differences in the molecular volume.

Fig. 5 shows the relationship between the permeability constant of aliphatic alcohol from ethanolic aqueous solution or ethanol solution through shed snake skin and the human skin cited from reference (Scheuplein & Blank 1971). These results show that permeability of shed snake skin to fatty alcohol is greater than that of human skin.

Esterase activity

Esterase activity in the shed snake skin was studied using *p*-nitrophenyl acetate as a model compound having an ester linkage in the molecule (Fig. 6). In *N. naja*, the apparent Michaelis–Menten constant (K_m) was 0·235 mM, and the apparent maximum velocity (V_{max}) was 0·0349 mM (mg protein)$^{-1}$ min^{-1}. From these results, *p*-nitrophenyl acetate would be significantly hydrolysed in the shed snake skin during the process of penetration. The hydrolysis decreased by 95% on heating the enzyme solution at 80°C for 15 min, demonstrating that the hydrolytic reactions are due to esterase in shed snake skin.

The effect of storage conditions on esterase activity was also studied. When shed snake skin was stored at 32°C, 70% r.h. for 5–6 months, K_m and V_{max} were 0·234 mM, 0·0214 mM (mg protein)$^{-1}$ min^{-1}, respectively. These values are not significantly different from initial values, suggesting that esterase activity of shed snake skin will be kept stable for up to 6

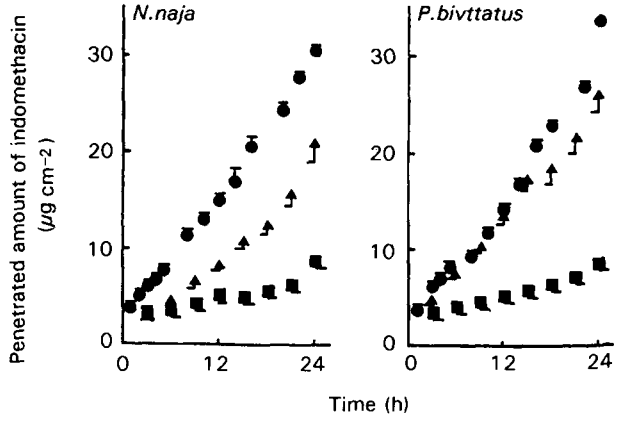

FIG. 4. Indomethacin penetration from a gel (1% indomethacin, ●), a cream (1% indomethacin, ■) or a liquid preparation (1% indomethacin, ▲) (all commercialy available) through shed snake skin. Results are represented as mean ± s.e.m. (n = 3).

Table 1. Effects of various penetration enhancers on the permeation of indomethacin through shed snake skin.

Enhancer	Flux of indomethacin ($\mu g\ h^{-1}\ cm^{-2}$)			
	Cream		Gel	
	N. naja	*P. bivttatus*	*N. naja*	*P. bivttatus*
Control	0·14 ± 0·01	0·68 ± 0·19	1·16 ± 0·01	1·66 ± 0·06
Azone (5%)	0·82 ± 0·10**	1·32 ± 0·05*	1·77 ± 0·10*	3·52 ± 0·17**
Dimethylsulphoxide (5%)	0·44 ± 0·11	0·70 ± 0·07	1·64 ± 0·10	2·08 ± 0·16
N-Methyl-2-pyrrolidone (5%)	1·60 ± 0·09**	0·83 ± 0·08	1·23 ± 0·21	1·66 ± 0·16
BL9-EX (5%)	0·21 ± 0·04	0·83 ± 0·03	0·91 ± 0·12	1·12 ± 0·30
Egg lecithin (5%)	0·46 ± 0·18	0·36 ± 0·03	1·23 ± 0·31	1·28 ± 0·10
N,N-Dimethyl-*m*-toluamide (5%)	0·80 ± 0·07**	3·28 ± 0·12	0·95 ± 0·10	0·99 ± 0·15

Results are represented as mean ± s.e.m. (n = 3). $^{**}P < 0.01$, $^{*}P < 0.05$ compared with control.

Table 2. Flux (J_s) and permeability constant (K_p) of aliphatic alcohols through shed snake skin.

Carbon number	1	2	3	4	5	6	7	8
N. naja J_s ($\mu mol\ h^{-1}\ cm^{-2}$)	132 ± 14·4	16·4 ± 1·37	5·45 ± 0·29 (2·82 ± 0·12)	3·61 ± 0·68 (1·35 ± 0·32)	2·10 ± 0·95	0·51 ± 0·12	0·43 ± 0·10	0·08 ± 0·01
P. bivttatus J_s ($\mu mol\ h^{-1}\ cm^{-2}$)	109 ± 3·09	14·4 ± 1·53	7·69 ± 1·56 (6·38 ± 0·45)	8·68 ± 2·17 (6·88 ± 0·45)	3·47 ± 1·82	2·21 ± 0·95	1·52 ± 0·55	0·15 ± 0·06
K_p in *P. bivttatus* ($cm\ h^{-1}$)			19·0	19·9	22·0	32·3	63·0	

Data are means ± s.e.m. (n = 3). Number in parentheses are J_s values of the branched alcohol.

months in these conditions. These results suggest that shed snake skin could be used as a model skin for preclinical screening of antedrugs which might be developed as locally acting drugs.

Discussion

In the present study, possibilities of the use of shed snake skin were examined for preclinical screening of useful formula for transdermal systemic or local drug delivery. Indomethacin and aliphatic alcohols were used as dissociable or non-dissociable model permeants, respectively. Indomethacin permeated through shed snake skin according to pH-partition theory in the range of pH 3-6.

Rigg & Barry (1990) reported that shed snake skin is more similar to human skin in terms of drug permeability than hairless mouse skin.

Hirvonen et al (1991) reported that rabbit pinna skin allowed permeation of model drugs more readily than human skin or shed snake skin and that the permeation of model drugs in shed snake skin was similar to that in human skin. Harada et al (1993) reported that permeation of salicylic acid through shed snake skin is comparable with the permeation in human chest and back skin. This report suggests that permeation of indomethacin through shed snake skin might be comparable with that in human skin, because indomethacin is an acidic compound like salicylic acid.

Shed snake skin has been reported to be similar to human stratum corneum in its ultrastructure (Landmann 1980, 1986; Landmann et al 1981) and total lipid content (Itoh et al 1990). However, Hirvonen et al (1993) reported that the surface charges are different from those in human skin. Furthermore, there are no appendageal penetration pathways in shed snake skin. Appendageal penetration pathways can be very significant in the early stages of penetration before steady-state diffusion is established (Scheuplein 1965, 1967). These observations may suggest the limitation of the use of shed snake skin as a model skin. As described earlier, model membranes corresponding to the different anatomical sites of the human body should be considered. Thus, shed snake skin could be used as a model for a particular site of human skin (Harade et al 1993). As to the effect of absorption enhancers on

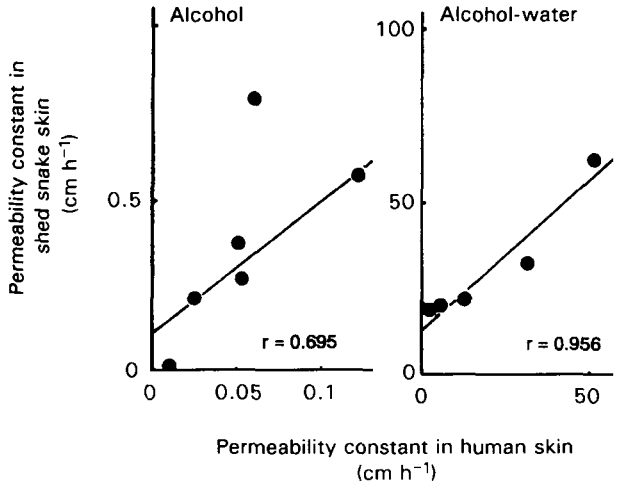

FIG. 5. Relationship of permeability constant (K_p) of aliphatic alcohols from pure alcohol and an alcohol–water system between human skin and shed snake skin (*P. bivttatus*).

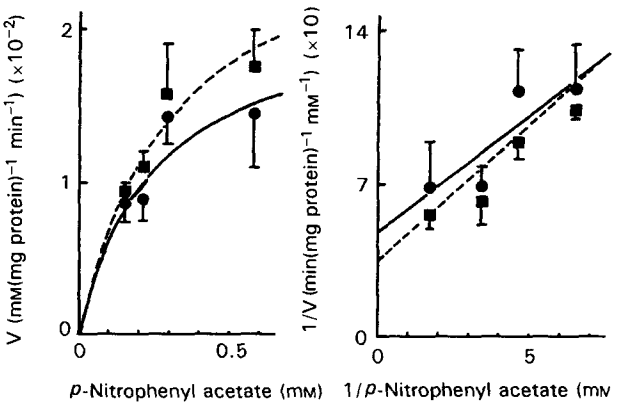

FIG. 6. Esterase activity in shed snake skin. *N. naja* ●; *P. bivttatus* ■. Results are represented as mean ± s.e.m. (n = 3).

indomethacin penetration, addition of Azone, *N*-methyl-2-pyrrolidone and *N,N*-dimethyl-*m*-toluamide to the cream preparation increased indomethacin penetration through *N. naja* shed snake skin. In the gel preparation only Azone enhanced indomethacin penetration through *N. naja* and *P. bivttatus* shed snake skin. The reason why the effects of penetration enhancers are different for the different dosage forms and species of shed snake skin is unclear.

In conclusion, the present study suggests that shed snake skin could be used as a model skin for preclinical screening of transdermal dosage forms and for estimating the efficiency of penetration enhancers.

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