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In-vitro Permeability to Salicylic Acid of Human, Rodent, and Shed Snake Skin

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Abstract—In-vitro permeability to salicylic acid of human, rodent, and shed snake skin has been examined for the purpose of selecting model membranes for human skin corresponding to different anatomic sites, since a marked regional variation is suggested among the different sites. The greatest permeability to salicylic acid was observed in the scrotum, that of the sole was negligible. The cheek, neck, and inguinal skin seemed more permeable than the breast, back, thigh, lower leg, or foot skin. Shed snake and skin of hairless rat were found to show similar permeability to human breast and thigh skin. Wistar rat and nude mouse skin showed similar permeability to human cheek, neck, and inguinal skin.

Transdermal systemic delivery of drugs is now clinically accepted for some drugs such as scopolamine, nitroglycerin, oestradiol and clonidine, since this has many advantages over the oral route (Guy & Hadgraft 1987). In particular, transdermal systemic delivery of drugs gives a sustained pharmacological action with less side-effects, and the drug delivery can be easily discontinued if adverse reactions occur.

In an in-vitro transdermal absorption study, the use of human skin would be preferred for predicting in-vivo transdermal drug delivery and for screening useful drug preparations. However, the use of human skin, including cadaver skin, is limited. Thus, selection of suitable model membranes is required for the in-vitro study of the transdermal absorption of drugs. Animal skins (Bond & Barry 1988; Hinz et al 1989) and artificial membranes (Houk & Guy 1988) have been examined as model membranes for human skin, and much information is available on species variation in skin permeability (Tregear 1966; Maibach et al 1971; Bartek et al 1972; Wester & Maibach 1975; Sinha et al 1978). It is also known that a marked regional variation exists in human skin permeability to hydrocortisone (Feldmann & Maibach 1967) and benzoic acid (Rougier et al 1986). Thus, different model membranes for human skin corresponding to different anatomic sites should be selected.

In the present study, the in-vitro permeability to salicylic acid was examined by using human skin excised from different anatomical sites, and rodent skin such as Wistar rat, hairless rat, and nude mouse skin. Shed snake skin was also used, since its usefulness as a model membrane for human skin has been suggested (Higuchi & Konishi 1987; Itoh et al 1990).

Materials and Methods

Materials

Salicylic acid was purchased from Wako Pure Chemicals Ltd (Osaka, Japan). Other chemicals used were of reagent grade and were used without further purification.

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Sources of skin

Human skin was obtained by surgical operation in Hiroshima University Hospital. Immediately after excision, subcutaneous fat was removed with tweezers and surgical scissors. The excised skin was kept at 4°C in sterilized 0·9% NaCl and was used within 24 h. For each human skin sample, anatomic region, age, and sex were recorded. In order to characterize the permeability of the stratum corneum, epidermis, and dermis, a full-thickness skin sample excised from the inner aspect of the thigh of a female (aged 42) was divided into three portions: one portion was separated into epidermis and dermis by immersing in hot water (60°C) for 30 s and then gently peeling off the epidermis in the same manner as reported by Swartzendruber et al (1987). No effect of immersing the skin in hot water on skin permeability to salicylic acid was observed in a preliminary experiment in which skin permeability was compared between untreated and hot water immersed full-thickness skin. The second portion of the skin was used after removing the stratum corneum by repeated stripping (20 times) of the stratum corneum with adhesive tape (Osamura et al 1984). Complete removal of the stratum corneum was confirmed by optical microscopic observation after haematoxylin-eosin staining. The third and remaining portion was used as an intact skin sample.

Male Wistar rats, 300–450 g, male hairless rats, 200–300 g, and male nude mice, 20–28 g, purchased from Hiroshima Laboratory Animal Company Ltd (Hiroshima, Japan), were anaesthetized with pentobarbitone (40 mg kg^{-1}). The dorsal skin hair was removed with clippers one day before the penetration study. The dorsal skin was excised, and underlying tissues were gently removed with tweezers and surgical scissors. Stripping the stratum corneum of the hairless rat skin sample was performed in the same manner as described for human skin.

Shed snake skin of the *Python reticulatus* was a gift from Dainihon Pharmaceutical Co. Ltd. It was stored at 70% relative humidity at room temperature (21°C). Shed snake

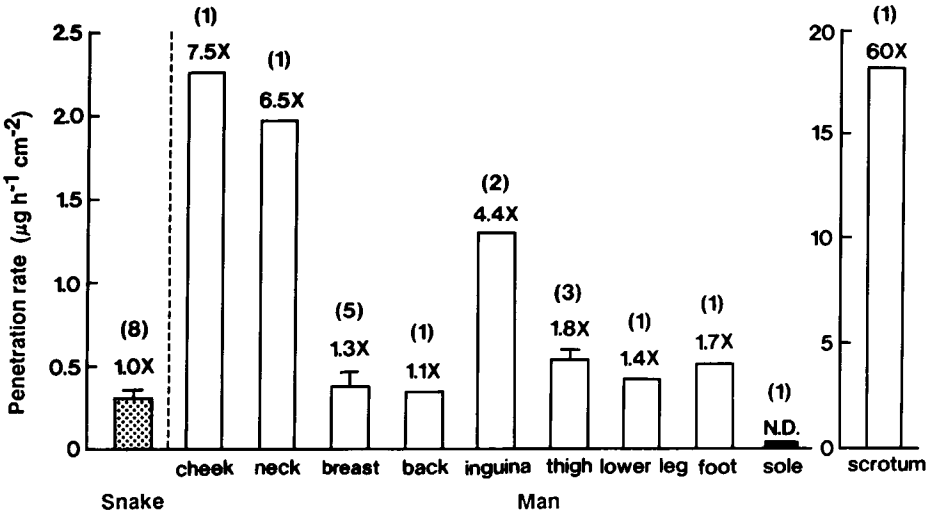

FIG. 1. Apparent penetration rate of salicylic acid at pH 4·0 in-vitro in shed snake skin and in intact human skin excised from various anatomic sites. The initial concentration of salicylic acid of the dosing solution was 500 μg mL^{-1} (1 mL). The number above the column denotes the relative permeability to salicylic acid of human skin against that of shed snake skin, and the number in parentheses represents the number of experiments. The vertical bar represents the s.e.m. N.D. indicates that penetration of salicylic acid was not detected during the 72 h experiment.

skin is a nonvital pure stratum corneum with no hair follicles, and consists of three distinctive layers of beta-, mesos- and alpha-layers (Landmann et al 1981). Each scale of the ventral skin was used after overnight hydration in an isotonic pH 7·4 Tris-HCl buffer before experimentation.

In-vitro penetration study

Permeability to salicylic acid of various skins was determined by using a Franz-type diffusion cell in a room with temperature maintained at 25°C. A circular piece of the skin was held securely between the two halves of the cell. The area of the skin exposed to the test solution was 0·785 cm^2 (1 cm diam.). Salicylic acid was dissolved (500 μg mL^{-1}) in isotonic buffer. The compositions of buffer solutions used were as follows: 0·1 M phosphate for pH 2·0; 0·1 M citric acid-phosphate for pH 3·0, 3·5 and 4·0; 0·05 M Tris-HCl for pH 7·4. One millilitre of salicylic acid solution was placed on the stratum corneum side in the upper donor cell, and the lower receiver cell was filled with 10 mL of isotonic pH 7·4 Tris-HCl buffer solution. The solution in the receiver cell was stirred vigorously with a magnetic stirring bar during each experiment to minimize the diffusion boundary layer at the skin interface. Samples of the solution (50 μL) in the receiver cell were withdrawn at designated time intervals until 72 h, at which time the same volume of the buffer solution was immediately resupplied. Aqueous samples were stored at −30°C until analysis. The penetration rate of salicylic acid through the skin was estimated from the slope of a regression line obtained by plotting the cumulative amount transported to the receiver side against time.

Transport resistance of the stratum corneum to salicylic acid

Transport resistance (R) of a skin to salicylic acid was estimated by the following equation:

R = concentration of salicylic acid in the donor cell (μg mL^{-1})/penetration rate of salicylic acid (μg h cm^{-2})

The R value of the stratum corneum was defined as the difference in R values between intact skin and stripped skin.

Analysis

To each aqueous sample (50 μL), acetonitrile (0·4 mL) and 0·1 M NaOH (1 mL) were added. The concentration of salicylic acid in the mixture was determined by fluorescence spectrophotometry (F-3000, Hitachi Ltd, Japan) at wavelengths of 298 nm for excitation and 407 nm for emission.

Results

Regional variation in human skin permeability to salicylic acid

The permeability to salicylic acid of human skin excised from

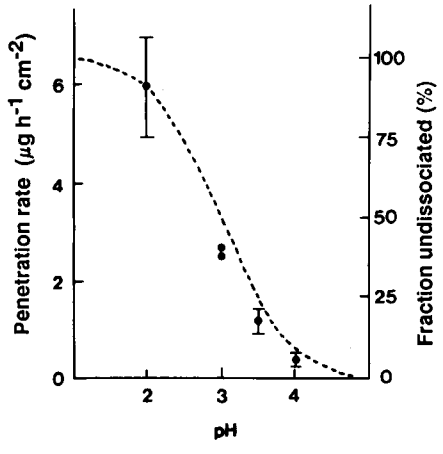

FIG. 2. pH-profiles of apparent penetration rate of salicylic acid through excised human breast skin in-vitro. The initial concentration of salicylic acid of the dosing solution was 500 μg mL^{-1} (1 mL). Each value represents the mean ± s.e.m. (n = 2–5). Breast skin was obtained from 5 donors. The dotted curve represents the theoretical value of undissociated fraction of salicylic acid.

Table 1. Penetration rate of salicylic acid through various excised skin.

pH	Apparent penetration rate (μg h cm^{-2})[a] Human (breast)[b]	Human (neck)	Snake	Hairless rat	Wistar rat	Nude mouse
2·0	5·97 ± 1·03	9·47, 11·1	5·22 ± 0·18	5·23 ± 0·50	12·41 ± 3·50	9·77 ± 0·13
3·0	2·49, 2·68	8·53	2·26 ± 0·05	3·65 ± 0·42	6·64 ± 1·18	5·81 ± 0·35
3·5	1·16 ± 0·28	4·14	0·81 ± 0·13	—	3·19 ± 0·14	—
4·0	0·37 ± 0·09	1·97	0·29 ± 0·05	0·66 ± 0·02	1·03 ± 0·07	1·60 ± 0·13
PR of undissociated form[c]	6·48	11·59	5·60	5·97	13·63	10·71
Correlation coefficient	0·997	0·951	0·993	0·985	0·999	0·999

[a] Each value represents mean ± s.e.m. of 3–9 trials. [b] Age of skin donor ranged from 38 to 74 years. [c] Penetration rate (PR) of undissociated salicylic acid calculated from the following equation by regression analysis; apparent PR = PR × undissociated fraction + PR of dissociated form.

different anatomic sites was determined at pH 4·0, which is close to the physiological pH of human skin surface. Salicylic acid penetrated the skin in a zero-order fashion following a lag time. Fig. 1 suggests a marked regional variation in salicylic acid permeability. In this study, the coefficients of intersubject variation in the penetration rate of salicylic acid were 24·0% in breast skin and 11·0% in thigh skin. Experimental variation among 3 specimens of breast skin obtained from one female was also examined. However, the variation coefficient in the 3 specimens was less than 1·5%. This low coefficient of variation in the 3 specimens indicates that the greater coefficient of variation observed in the breast and thigh skin may be due to intersubject variation. This intersubject variation may result from the differences in the thickness of the stratum corneum and the total lipid content in the stratum corneum (Elias et al 1981). Since the results in cheek, neck, back, lower leg, foot, sole, and scrotum (Fig. 1) are the product of one experiment each, these data could not be used in a statistical comparison. However, extraordinarily greater permeability of the scrotum and negligible permeability of the sole to salicylic acid is apparent. The face, neck, and inguinal skin seem more permeable than the breast, back, thigh (inner aspect), lower leg (pretibial), and foot (dorsal). The penetration rate of salicylic acid through shed snake skin is also shown (Fig. 1). The relative permeability to salicylic acid of human skin against that of shed snake skin (indicated by the numbers above each column) shows that shed snake skin could be used as a model membrane for human breast and thigh skin.

The effect of age of the skin donor on the permeability to salicylic acid was evaluated using breast skin of 5 different female donors (38–74 years). However, no significant relationship between the penetration rate and age was observed.

Species variation in skin permeability

A typical example of the pH profile of the apparent salicylic acid penetration rate is shown in Fig. 2, using human breast skin from a total of 5 female donors (38–74 years). The apparent penetration rate increased with an increase of the undissociated fraction in the dosing solution. Similar pH-dependent penetration was observed in shed snake, hairless rat, Wistar rat, and nude mouse skin. Species variation in skin permeability was examined by estimating the undissociated salicylic acid penetration rate by plotting apparent penetration rate at each pH against the undissociated fraction of salicylic acid in the dosing solution. A linear relationship was observed, indicating that the physiological effect of a high acidity on skin permeability is negligible. The value of the intercept of the line represents the theoretical penetration rate of dissociated salicylic acid. However, the observed value was not significantly different from the origin. Thus, we concluded that penetration of dissociated salicylic acid was negligible. The penetration rate of undissociated salicylic acid estimated from the slope of the line, is summarized in Table 1. Shed snake and hairless rat skin were found to show similar permeability to human breast skin at each pH, whereas Wistar rat and nude mouse skin showed similar permeability to human neck skin.

Barrier function of the stratum corneum

Full-thickness human skin and rodent skin have underlying tissues of viable epidermis, dermis, and stratum corneum, which is different from the structure of shed snake skin. Thus, the barrier function of the stratum corneum itself to salicylic acid penetration was assessed by using human thigh skin and hairless rat skin.

Fig. 3 shows cumulative amounts of salicylic acid which penetrated through full-thickness human thigh skin, epidermis, dermis, and tape-stripped skin. The apparent penetration rate in full-thickness skin at pH 2·0 or 4·0 was almost equal to that through the epidermis at each corresponding pH, although the lag time in full-thickness skin was about 5–10 h longer than that of epidermis. However, the skin without stratum corneum (dermis and stripped skin) was much more permeable than full-thickness skin and epidermis. Additionally, the effects of the dosing solution pH on the apparent penetration rate completely disappeared in dermis and tape-stripped skin. Similar results were also observed in tape-stripped hairless rat skin. These results indicate that the barrier function to salicylic acid penetration exists mainly in the stratum corneum.

The transport resistance of the stratum corneum to salicylic acid in human thigh skin and hairless rat skin was calculated by subtracting the transport resistance in tape-stripped skin from that in full-thickness skin (Table 2). Transport resistance through shed snake skin is also listed in Table 2. The transport resistance of the stratum corneum in human thigh skin and hairless rat skin increased with an increase of dissociated fraction of salicylic acid in the dosing solution. Their transport resistance values were comparable

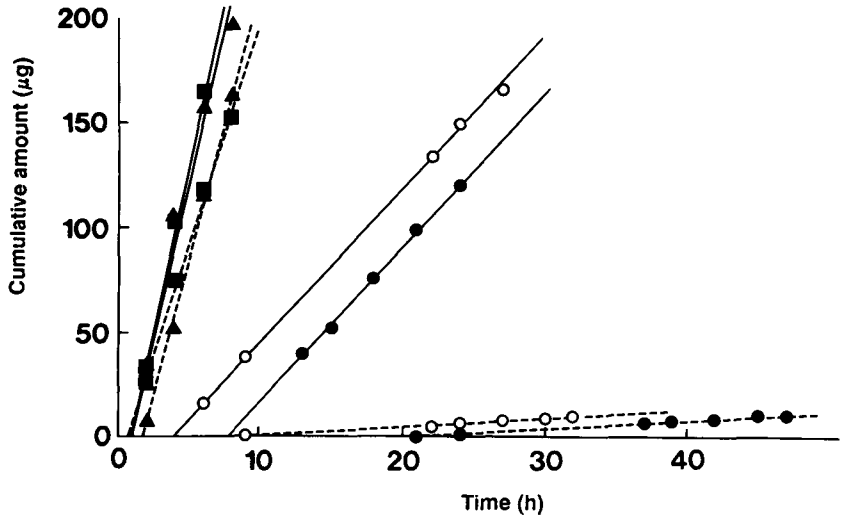

FIG. 3. Penetration of salicylic acid through full-thickness human skin (●), epidermis (○), dermis (■), and stripped skin (▲) in-vitro. The initial concentration of salicylic acid in the dosing solution was 500 μg mL^{-1}. The skin was excised from the inner aspect of the thigh of a 42 years old female. Solid and dotted lines represent the results at pH 2·0 and 4·0, respectively. The line was obtained by regression analysis.

with those of shed snake skin. These results indicate that the stratum corneum of human thigh skin and hairless rat skin has a barrier function similar to that of shed snake skin.

Discussion

In-vitro permeability to salicylic acid of human, rodent, and shed snake skin was examined for the purpose of selecting suitable model membranes for human skin. The skin permeability study was conducted at 25°C, since the integrity of the skin was found to be maintained for a longer time at 25°C compared with 32 or 37°C in a preliminary experiment. Also, it has been reported that a linear relationship exists between the skin penetration rate and experimental temperatures ranging from 25 to 50°C (Blank et al 1967).

As shown in Table 2, the stratum corneum is the main permeability barrier. It has been reported that underlying tissues (viable epidermis and dermis) create another diffusion barrier for especially lipophilic compounds (Bronaugh 1989) and these underlying tissues provide transport resistances, especially at low pH; however, the contribution of the underlying tissues to the whole transport resistance decreased with an increase of dissociated fraction of salicylic acid in the dosing solution (Table 2). We, therefore, chose to study human skin permeability at pH 4·0.

Table 2. Transport resistance of the stratum corneum of human breast skin, skin of hairless rat and shed snake skin in penetration of salicylic acid.

	Transport resistance (h cm^{-1})[a]		
pH	Human (breast)	Hairless rat	Snake
2·0	63·0 ± 17·2 (0·723)[b]	70·9 ± 8·10 (0·711)	95·8 ± 2·70
3·0	Not determined	123 ± 16·9 (0·851)	221 ± 53·3
4·0	1340 ± 406 (0·975)	727 ± 27·8 (0·963)	1720 ± 2·70

[a] Each value represents the mean ± s.e.m. of 3–5 trials. Transport resistance = concn of drug in donor cell (μg mL^{-1})/penetration rate of drug (μg h cm^{-2}). [b] Ratio of transport resistance of the stratum corneum to that of whole skin.

Rougier et al (1986) reported that the penetration of benzoic acid through human skin increased in the order: back < arm < chest < thigh < abdomen < forehead, with the penetration in the forehead being 3 times greater than in the back. Feldmann & Maibach (1967) reported that the extent of apparent transdermal absorption of hydrocortisone in-vivo was high in areas with follicles (forehead, scalp, and scrotum) and low in the areas where the stratum corneum is relatively thick (sole and palm). Conclusions almost similar to those of Feldman & Maibach (1967) were obtained in the present study using salicylic acid (Fig. 1). However, in the present study, strict ordering of permeability of different anatomic sites was difficult because of an insufficient number of experimental data in some anatomic sites and possible intersubject variation (Fig. 1).

The usefulness of shed snake skin as a model membrane for human skin has been previously reported (Higuchi & Konishi 1987; Itoh et al 1990). Our data resulted in a similar conclusion, but the usefulness of shed snake skin may be restricted to human breast and thigh skin. Shed snake skin has been reported to be similar to the human stratum corneum in its ultrastructure (Landmann 1980, 1986; Landmann et al 1981) and total lipid content (Itoh et al 1990), although their lipid components are markedly different. The main polar lipids are phospholipids in shed snake skin (Roberts & Lillywhite 1980) and ceramides in man (Long et al 1985). Thus, our data also may support the suggestion by Elias et al (1981) that total lipid content, rather than lipid composition, plays the more important role in barrier functions of the stratum corneum. In the present study, the contribution of hair follicles in salicylic acid penetration through human skin was not well recognized.

In conclusion, shed snake skin (scale in ventral site of *Python reticulatus*) and skin of hairless rat were found to show similar permeability to that of human breast and thigh

skin. The dorsal skin of the Wistar rat and nude mouse showed similar permeability to that of human cheek, neck, and inguinal skin. These findings will be a useful guide for selecting suitable model membranes for human skin of different anatomic sites.

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