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Shed king cobra and cobra skins as model membranes for in-vitro nicotine permeation studies

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Abstract

Shed king cobra skin (SKCS) and shed cobra skin (SCS) were investigated for use as barrier membranes, including some pre-hydration factors, for in-vitro nicotine permeation. Inter-specimen variations in nicotine fluxes using shed snake skin were compared with those using human epidermis. Nicotine in the form of 1 % w/v aqueous buffer solution at pH 5 and transdermal patches (dose 14 mg day^{-1}) were used. The nicotine fluxes across the shed snake skin were not significantly affected ($P > 0.05$) by temperature and duration of hydration pre-treatment. Scanning electron micrographs of SKCS and SCS revealed a remarkable difference in surface morphology, but the nicotine fluxes using both shed skins were not significantly different ($P > 0.05$). When compared with the results obtained using human epidermis, there were similarities in fluxes and permeation profiles of nicotine. Using nicotine solution, the nicotine permeation profiles of all membranes followed zero order kinetics. The amount of nicotine permeated provided good linearity with the square root of time over 24 h ($R^2 > 0.98$) when using nicotine patches. The nicotine fluxes using SKCS and SCS had less inter-specimen variation than those using human epidermis. The results suggest a potential use for SKCS or SCS as barrier membranes for in-vitro nicotine permeation studies.

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Introduction

A barrier membrane is essential in in-vitro permeation studies. Although human cadaver skin is mostly used as a model membrane, there is a limitation to its availability and variability (Itoh et al 1990a). Some animal skins have been proposed for use as alternative in-vitro permeation membranes. However, most animal skins allowed greater permeability than human skin (Bartek et al 1972; Wester & Noonan 1980; Itoh et al 1990a, b; Harada et al 1993).

Shed snake skin of the black rat snake (*Elaphe obsoleta*) has been widely used in permeation studies of drugs and in the investigation of the effects of several permeation enhancers (Hirvonen et al 1991; Takahashi et al 1993; Suh & Jun 1996; Takahashi & Rytting 2001). Shed snake skin is not a living tissue, consists of three distinctive layers, beta-, mesos- and alpha-layers, and lacks hair follicles (Itoh et al 1990a). The main polar lipids in shed snake skin are phospholipids, whereas those in human skin are ceramides (Harada et al 1993). Despite the obvious differences in anatomy between human skin and shed snake skin, the permeation fluxes of some drugs across shed snake skin showed good correlation with those of human skin (Itoh et al 1990a).

Nicotine (MW 162.2) is highly soluble in lipids and aqueous solvents. The permeation of nicotine has been studied using several membranes, such as hairless rat skin (Ho & Chien 1993), pig skin (Nair et al 1997), Göttingen minipig skin (Qvist et al 2000) and human skin (Oakley & Swarbrick 1987). Nicotine transdermal delivery devices have been developed for use in nicotine replacement therapy (Berner et al 1992; Fang et al 1999). A nicotine transdermal patch was prepared and evaluated in our laboratory. The patch was shown to be an effective device for nicotine delivery in permeation studies using human skin and whole cobra skin as barrier membranes (Pongjanyakul et al 2000). Shed snake skin may be an alternative to animal skin since it can be obtained periodically and repeatedly from the same snake without injury to the snake. This

membrane also provides a good opportunity for reducing inter-individual variability. King cobra (*Ophiophagus hannah*) and cobra (*Naja Naja Khaotia*) may be more readily available than black rat snakes in Thailand. We therefore intended to investigate the use of shed king cobra skin (SKCS) and shed cobra skin (SCS) as barrier membranes for nicotine. We report the effects of temperature and duration of hydration pre-treatment on nicotine permeation through SKCS and SCS using a factorial design. The inter-specimen variation of both types of shed snake skin compared with human skin was also investigated.

Materials and Methods

Materials

(−)-Nicotine and methanol, high-performance liquid chromatography (HPLC) grade, were purchased from Fluka (Buchs, Switzerland), and Baker Incorporated (Phillipsburg, NJ, USA), respectively. Nicotinell (dose 14 mg day^{-1}, Lot no 236600) was purchased from Novartis (Thailand) Ltd (Bangkok, Thailand) and used as supplied. Other reagents used were of analytical grade and used as received.

Skin preparation

SKCS and SCS were gifts from the King Cobra Village, Khon Kaen, Thailand and the Thai Red Cross, Bangkok, Thailand, respectively. They were washed with water, dried at room temperature and then stored frozen (−20°C) until use (Itoh et al 1990a; Hirvonen et al 1991). Before use, the dorsal portion of the shed snake skin was cut into an appropriate size, rinsed several times with distilled water and then hydrated using phosphate buffer (pH 7.4).

The abdominal cadaver skin of Thai males (46–50 years old) was obtained from the Department of Pathology, Faculty of Medicine, Khon Kaen University, within 24 h of death. The skin selected for use had minimal visible hair. The human epidermis was separated from the whole skin after soaking in distilled water at 60°C for 2 min and then stored at −20°C until use. Before use, the human epidermis was hydrated by soaking in phosphate buffer (pH 7.4).

Factorial design study

Before permeation studies, the barrier membrane is normally pre-treated by hydration. Different studies have used different hydration conditions, including temperature and duration (Itoh et al 1990a; Harada et al 1993; Takahashi & Rytting 2001). In the present study, the effects of temperature and duration of the hydration pre-treatment of shed snake skin before permeation testing were investigated. A factorial design with each factor at two levels was performed: temperature: low level, 25–27°C (room temperature), high level, 37°C; duration: low level, 0.5 h, high level, 12 h. Four experiments for a 2^2 factorial design were conducted on both shed snake skins using 1% w/v nicotine in phosphate buffer solution (pH 5) in the donor compartment.

Permeation studies

A 6-mL modified Franz-diffusion cell (diam. 1 cm) was used. Each of the cells was stationed in a cell-mounting block having a receptor compartment that was thermostatically maintained at 37°C. The receptor medium, phosphate buffer (pH 7.4), was constantly stirred at 600 rev min^{-1}. A piece of the pre-treated skin was mounted on a diffusion cell, with the stratum corneum surface facing the donor compartment. The donor compartment was filled with 1 mL 1% w/v nicotine in phosphate buffer solution (pH 5) or a circular nicotine patch with a diameter of 0.84 cm. The cells were then fixed and tightly fastened by a clamp. Samples (0.4 mL) were collected at various times and replaced with fresh phosphate buffer (pH 7.4). The concentration of nicotine in the receptor compartment was analysed by HPLC.

HPLC analysis of nicotine

The concentration of nicotine permeated was determined using HPLC (Perkin Elmer, USA). A reversed-phase HPLC using a C-18 column (Spherisorb ODS-2, 5 μm, 4.6 × 250 mm) was employed. The mobile phase was 0.05 M sodium acetate/methanol (88:12 v/v) containing 0.5% triethylamine; the pH was adjusted to 4.2 with glacial acetic acid. The HPLC conditions were as follows: flow rate, 1 mL min^{-1}; UV detection at 259 nm; paracetamol as internal standard. The retention times of nicotine and paracetamol were approximately 5.3 and 8.4 min, respectively. Under these conditions, good linearity and reproducibility were shown over the range 1–400 μg mL^{-1} nicotine free base.

Scanning electron microscopy (SEM)

The surface morphology of the shed snake skin was investigated using SEM. Samples were mounted onto aluminium stubs, sputter coated with gold in a vacuum evaporator, and photographed using a Jeol Model JSM-5800LV SEM.

Data analysis

Steady-state fluxes of the permeation of nicotine across human epidermis and shed snake skin can be calculated using linear regression analysis of the linear relationship between the amount of nicotine permeated and time. This relationship can be described by using Fick's first law (Flynn et al 1987) as follows:

$$dM/dt = AP(\Delta C) \quad (1)$$

where dM/dt is the permeation rate, A is the surface area of the skin through which the diffusion is taking place, dM/dt × A is the permeation flux, ΔC is the concentration difference across the half-cell (usually approximated by the nicotine concentration in the donor compartment) and P is the permeability coefficient.

Analysis of variance (SPSS program for MS Windows, release 8.0) was used to determine significant differences between permeation data.

Results and Discussion

Surface morphology of shed snake skin

The shed snake skin could be divided into two defined regions: scale and hinge (scales are separated by hinges). The scales of shed snake skin are rigid, whereas the hinges are elastic. The hinge is thinner than the scale (Haigh et al 1998). The scale size of SKCS was somewhat larger than that of SCS. The surface morphology of scales and hinges of a dorsal portion of the shed snake skin, studied using SEM, is shown in Figure 1. The structure of the scale and hinge was microscopically different. Comparison of SEM images of the SCS scale (Figure 1A) with that illustrated by Haigh et al (1998) indicated a similar folded pattern, although different species were used. The folded pattern of the scale of SKCS suggested a rougher surface (Figure 1B). Moreover, the hinge structure of SKCS and SCS also showed obvious differences (Figure 1C and D).

Effect of hydration pre-treatment on nicotine permeation

Permeation behaviour of nicotine across shed snake skin followed Fick's first law (equation 1) ($R^2 > 0.99$). The nicotine fluxes using 1% w/v nicotine in phosphate solution at pH 5 across both SKCS and SCS under various conditions are presented in Table 1. The mean nicotine fluxes over 24 h increased with increasing temperature and duration of hydration pre-treatment. However, the hydration pre-treatment factors showed no significant effect ($P > 0.05$) on the nicotine fluxes across the shed snake skins, or statistically mutual interactions ($P > 0.05$). The results suggested that the optimal hydration pre-treatment

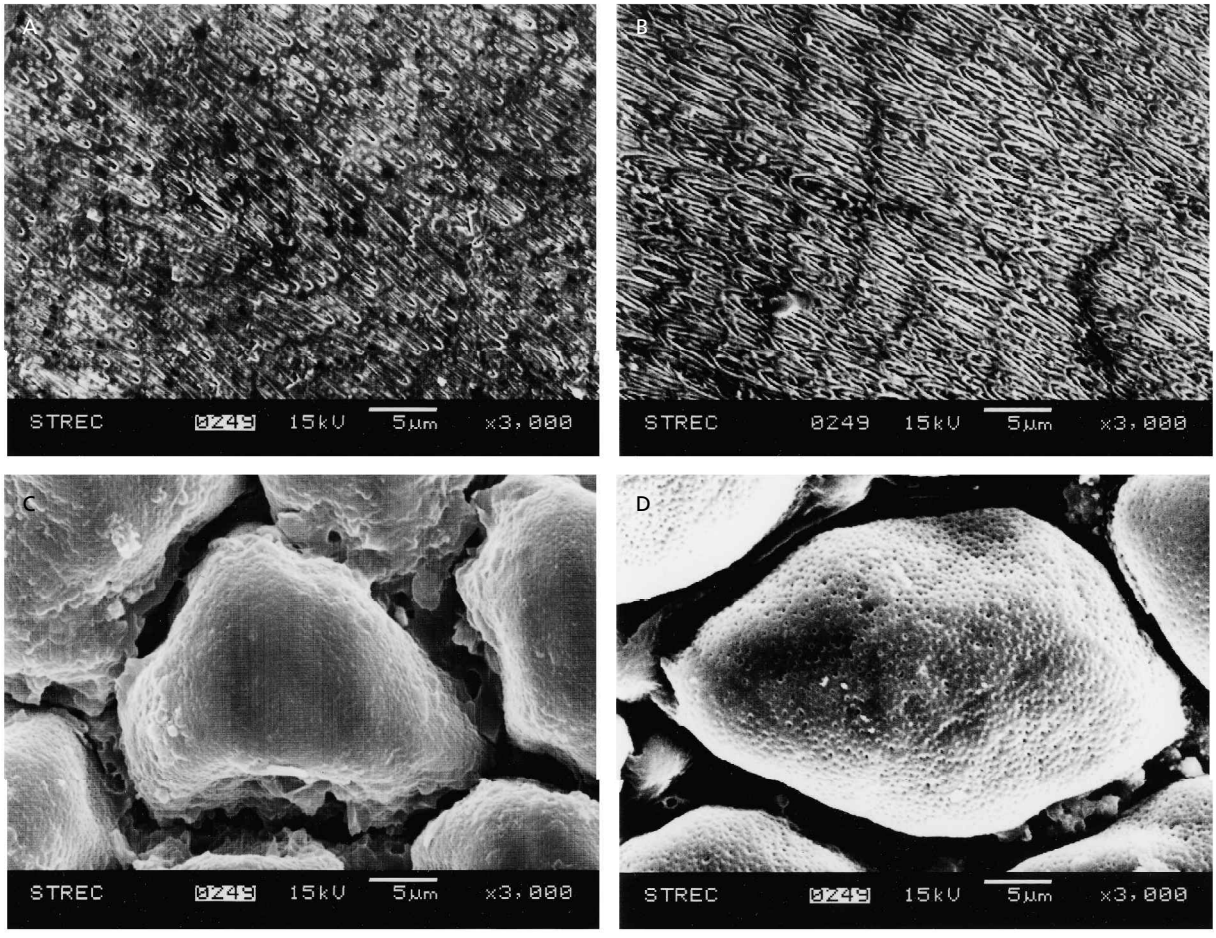

Figure 1 Surface morphology of a dorsal portion of shed snake skin: scale of cobra (A) and king cobra (B); hinge of cobra (C) and king cobra (D).

Table 1 Effect of hydration pre-treatment before permeation studies on nicotine flux.

Temperature (°C)	Duration (h)	Nicotine flux (μg cm^{-2} h^{-1})	
		Shed king cobra skin	Shed cobra skin
25–27	0.5	4.16±1.1	4.46±0.8
	12	4.67±0.7	4.66±0.8
37	0.5	4.50±0.7	4.57±0.4
	12	5.08±1.0	4.70±0.9

Data are means±s.d., n = 3.

of the shed snake skins was 0.5 h at a temperature range of 25–27°C. These were the pre-treatment conditions used for the permeation study.

Nicotine permeation study

The fluxes of 1% w/v nicotine solution across SKCS and SCS from five snakes were over the range 2.90–4.16 and 3.63–4.74 μg cm^{-2} h^{-1} (n = 3 each), respectively. The coefficient of variation (CV) was 6.78–18.6% for SCS and 8.57–26.7% for SKCS. The fluxes obtained for SKCS and SCS from five snakes were significantly different ($P < 0.05$), indicating the inter-specimen variation of the skins. The mean nicotine fluxes were higher across human epidermis than those across shed snake skins (Table 2). The mean permeability coefficient of nicotine using human epidermis as the barrier membrane was 1.7- and 1.4-times those using SKCS and SCS, respectively. As expected, using human epidermis, a longer lag time and a greater inter-specimen variation of permeation flux were observed (Table 2). The results obtained using shed snake skin were in agreement with those reported for shed black rat snake skin using methylparaben (CV 31.4%) and corticosterone (CV 40.2%) (Itoh et al 1990a). An advantage of using shed snake skin is the lower inter-specimen variation of the permeation results in-vitro.

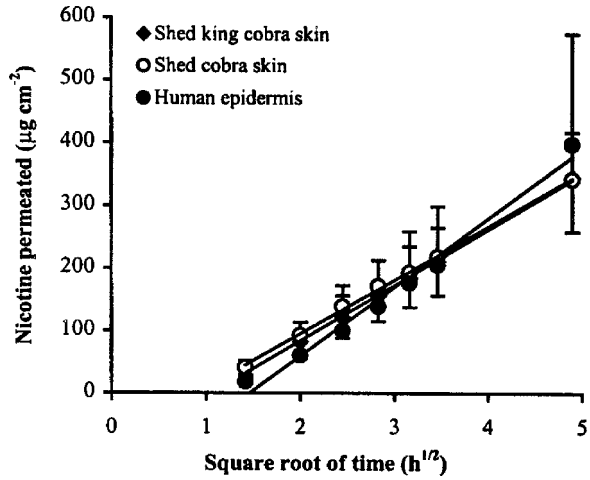

Figure 2 Relationship between the amount of nicotine permeated and the square root of time across various types of membranes using transdermal patches. Points are means±s.d. of seven determinations for human epidermis (three specimens) and 15 determinations for shed king cobra skin and shed cobra skin (five snakes).

The permeation profiles of nicotine across three types of membranes using the transdermal patch did not follow zero order kinetics. However, the nicotine permeated across all membranes showed a good linearity with the square root of time ($R^2 > 0.98$) when investigated using linear regression analysis (Figure 2). This indicated that the matrix skin system formed a single homogenous polymeric film. The results were similar to those using whole cobra skin as a membrane in a previous study (Pongjanyakul et al 2000). The equation used to describe the transport of nicotine across the three types of membrane is as follows (Kydonieus 1987):

$$M_t = 4M_\infty(Dt/\pi L^2)^{1/2}, \quad 0 \leqslant M_t/M_\alpha < 0.6 \qquad (2)$$

where M_t is the amount of drug released at a given time, M_∞ is the amount of drug released at infinite time, D is the diffusion coefficient of the drug in the matrix, L is the thickness of the matrix, and t is time. An approximation of

Table 2 Permeation parameters of nicotine across various types of membranes.

	Human epidermis	Shed king cobra skin[a]	Shed cobra skin[a]
1% w/v nicotine solution			
Flux (μg cm^{-2} h^{-1})	5.87±2.0[b] (CV 34.1%)	3.49±0.8 (CV 22.9%)	4.24±0.7 (CV 16.5%)
P (cm s^{-1} ×10^7)	1.63±0.6[b]	0.97±0.2	1.18±0.2
Mean lag time (h)	2.11	0.55	0.74
Nicotine patch			
Flux (μg cm^{-2} h$^{-1/2}$)	110.2±46.7[c] (CV 42.4%)	89.0±23.0 (CV 25.8%)	86.0±19.5 (CV 22.7%)
Mean lag time (h)	1.04	0.26	0.07

[a]n = 15 from five snakes. [b]n = 6 from three specimens. [c]n = 7 from three specimens. CV, coefficient of variation; P, permeability coefficient.

the equation could be shown by plotting the cumulative amount of nicotine permeated per unit area and the square root of time ($t^{1/2}$) as presented by equation 2:

$$M_t = kt^{1/2} \quad (3)$$

where k is the nicotine permeation flux.

The nicotine fluxes across SKCS and SCS (five snakes; n = 3 each) using transdermal patches were 60.4–108.6 and 65.4–107.3 $\mu g\ cm^{-2}\ h^{-1/2}$, respectively. The CV of nicotine permeation fluxes for SCS and SKCS using transdermal patches was over the range 7.04–29.8 and 7.23–25.7%, respectively. The inter-specimen variation of these shed snake skins gave similar results when compared with using 1% w/v nicotine solution. The mean nicotine fluxes across the three types of membrane showed a higher inter-specimen variation when using human epidermis than those using shed snake skin (Table 2). The nicotine flux across human epidermis was 1.2- and 1.3-times higher than that across SKCS and SCS, respectively. The difference in nicotine permeation flux between human epidermis and shed snake skins using transdermal patches was smaller than that using nicotine solution. A decrease in the ratios of the permeation fluxes could be attributed to the controlled release of nicotine from the patch.

Inter-specimen variation of human epidermis was higher than that of shed snake skin. This result was similar to previous reports (Southwell et al 1984; Pongjanyakul et al 2000). Human skin had greater inter-specimen variation because of the variation of hair follicles (Bialik et al 1993) and the thickness of epidermis (Whitton & Everall 1973). In the case of shed snake skins, with careful selection of the membrane it may possible to control the key factor affecting the diffusion of nicotine, that is the scale to hinge ratio (Haigh et al 1998). This explains the variation of nicotine fluxes when using the shed snake skins.

The mean lag times of nicotine across both shed skins were not significantly different. This finding is in agreement with the findings reported by Haigh et al (1998). The mean lag times of nicotine across shed snake skins were found to be shorter than that across human epidermis. These results agree with previous reports that the lag time of neutral and acidic compounds with molecular weight below 200 (Itoh et al 1990a, b) and basic compounds (Takahashi et al 1993) were not observed when using shed snake skin from black rat snake. Furthermore, the partition coefficient of nicotine (octanol/buffer solution at pH 5.4) is approximately 0.3 (Nair et al 1997). This suggested a lipophilic property of nicotine that could partition reasonably well into the skin (Guy & Hadgraft 1989).

Conclusion

The fluxes and permeability coefficient of nicotine across the SKCS and the SCS were similar to those across human epidermis. The permeation profiles of nicotine from the patches across shed snake skins and human epidermis had identical kinetic profiles, showing linear relationships between the nicotine permeated and the square root of time. Moreover, the nicotine fluxes across shed snake skins using nicotine in the form of a solution and patch provided a lower inter-specimen variation compared with using human epidermis. Thus, SKCS and SCS have potential use as barrier membranes for in-vitro nicotine permeation studies.

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