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Research paper

Comparison of skin transport and metabolism of ethyl nicotinate in various species

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Abstract

The skin transport and metabolism characteristics of ethyl nicotinate (EN) in rabbit, rat, guinea-pig, pig, shed snake skin and human were compared. In vitro skin transport using excised skin and hydrolysis experiments using skin homogenate were carried out. Flux of EN, a metabolite, nicotinic acid (NA), and the total (EN + NA), as well as kinetic parameters (V_{max} and K_m) for hydrolysis of EN were determined and compared among various species. The enzymatic conversion of EN to NA was observed for all skin permeation experiments. Total flux from EN-saturated solution between rabbit, rat, guinea-pig and human was significantly different ($P < 0.05$). A great difference between species was observed in skin esterase activity. The NA/total flux ratio of human was significantly lower than that of rabbit, rat or guinea-pig but lower than that of shed snake skin ($P < 0.05$). There is no significant difference in skin permeation and metabolism between human and pig ($P > 0.05$). Total flux increased linearly with an increase in EN donor concentration for all species. For pig, shed snake skin and human, NA flux increased with an increase in EN donor concentration and reached a plateau, suggesting the metabolic saturation was taking place in the skin. NA flux at plateau and EN donor concentration in which the NA flux reached a plateau were also affected by species difference. These findings indicated that the discrepancy in transdermal profiles of EN among species tested was predominantly due to the difference in the esterase activity in the skin.

Keywords: Species difference; Skin transport; Skin metabolism

1. Introduction

The utilization of lipophilic prodrugs is a useful method to enhance the transdermal absorption of therapeutic agents [1,2]. Dermal enzymes can convert prodrugs into pharmacological active forms, and also inactivate or detoxify xenobiotics [3]. To evaluate the effectiveness of the prodrugs on the skin, simultaneous skin permeation and metabolism must be studied. Theoretically, such study should use humans for testing. However, ethical considerations are the major problems in using human skin as a model membrane. Therefore, animal skins such as rat, rabbit, guinea-pig, pig and shed snake skin are frequently used as an alternative because they are easy to handle, lower in cost, and lower in variation in permeability from one specimen to other [4–6].

The species difference in skin permeability of drugs have been extensively reported, however, very little data is available comparing the skin metabolism in mammalian skin and shed snake skin with that of human skin [7,8]. The extent and rate of skin metabolism have been found to affect the skin permeability of prodrugs [9,10]. The prodrugs should be metabolized completely in the skin layer to maximize topical therapeutic activity and to minimize both topical and systemic side effects. Knowledge of the comparative qualities in skin metabolism and permeation between different species is important as it can be applied to estimate the pharmacological and adverse effects of prodrug therapy in humans by prediction from the animal results.

The present study focused on the comparison of simultaneous skin transport and metabolism of prodrug between different species. Ethyl nicotinate (EN) was selected as a model prodrug of nicotinic acid (NA) because

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it can be hydrolyzed by esterases in the skin [9,11]. In vitro skin permeation characteristics and enzyme activity in skin homogenate were compared among human, rabbit, rat, guinea-pig, pig and shed snake skin.

2. Materials and method

2.1. Materials

NA and EN were obtained from Tokyo Chemical Industries (Tokyo, Japan). All other reagents and solvents were of analytical grade and obtained commercially.

2.2. Preparation of membranes

The method of the percutaneous absorption study followed Test Guideline 428 of Organization for Economic Cooperation and Development (OECD) [12]. Male Wistar rat (200–250 g), guinea-pig (260–350 g) and Albino rabbit (2500–4500 g) were supplied by the National Animal Center, Mahidol University (Bangkok, Thailand). The abdominal skin was carefully shaved and freshly excised before the experiments under pentobarbital anesthesia (50 mg/kg, i.p.), and cervical dislocation. The abdominal skin of new born (0–1 day) pig (*Sus scofa*) was obtained immediately after slaughter in the general slaughterhouse (Nakorn Pathom, Thailand). Shed snake skin of *Elaphae obsoleta* was kindly donated by the Saowabha Institute (Bangkok, Thailand). After the skin was obtained, it was stored at −20 °C prior to use. The original esterase activity in shed snake skin was reported to be the same, even the skin was kept at this condition for 6 months [8].

Human skin was obtained following unrelated surgical operations (Department of Surgery, Yanhee General Hospital, Bangkok, Thailand). The source was the breast of female patients (35–67 years old). The skin was stored at −20 °C prior to use in order to maintain the original activity of skin enzymes [13]. The samples were gradually thawed in 0.9% (w/v) NaCl solution, and were prepared to be split-thickness skins (0.6–0.7 mm) by dermatome.

2.3. Skin transport experiment

The skin samples were mounted between two half-cells of a side-by-side diffusion chamber (3.0 ml volume and 0.966 cm^2 effective diffusion area) with a water-jacket connected to a water bath at 37 °C. The dorsal surface of the skin was placed in contact with the drug solution. The receiver and donor compartments were filled with 0.1 M phosphate buffered saline (PBS) (pH 7.4) and stirred at 1440 rpm with a star-head Teflon® magnetic bar (Nalge Nunc International Co. Ltd, MN, USA) driven by a synchronous motor. After 1 h equilibration, the media in the receiver and donor compartments was replaced with fresh PBS and various concentrations of EN (6–244 mM) in PBS, respectively. A part of the receiver solution (0.5 ml) was collected every hour for 8 h, and the same volume of fresh PBS was replaced to keep the volume constant. The samples were stored at 4 °C until analyzed. The cumulative amount of EN, NA and the total (EN + NA) was plotted against time, and the flux at pseudo-steady-state was determined from the slope of linear regression analysis.

2.4. Hydrolysis of ethyl nicotinate in skin homogenate

The kinetic analysis for enzymatic hydrolysis of EN was performed using skin homogenate. Skin homogenate (25%, w/w) was prepared with full-thickness skin freshly excised from various ages of rats and PBS using a tissue homogenizer. The homogenate was centrifuged for 10 min at $9000 \times g$ and 4 °C. The supernatant and various concentrations of EN in PBS were preincubated for 15 min, then the prepared skin homogenate was mixed to make final concentration of 5% (w/w) homogenate and maintained at 37 °C. Samples were taken at an appropriate time point, and the enzymatic reaction was terminated by adding the same volume of acetonitrile. The chemical stability of EN was also evaluated in enzyme-free PBS at 37 °C. All samples were kept at 4 °C until analyzed. The hydrolysis rate was determined by the slope of the plot between the remaining content of EN and incubation time. The Michaelis–Menten parameters (maximum hydrolysis rate, V_{max}; and Michaelis–Menten constant, K_m) were determined by the Lineweaver–Burk plot. This plot is a derivation of the Michaelis–Menten equation

$$1/V_0 = 1/V_{max} + K_m/V_{max}[S] \quad (1)$$

where V_0 is the hydrolysis rate and [S], the EN concentration. The intercept on the $1/V_0$-axis and 1/[S]-axis equals to $1/V_{max}$ and $1/K_m$, respectively. The protein content in skin homogenate was determined by Lowry's method [14]. The values of V_{max} were expressed in two methods: in unit of nmol/min per mg protein, and in unit of μmol/cm^2 per hour using the values of protein content of 5% (w/w) skin homogenate and weight per area of skin. The intrinsic metabolic clearance (V_{max}/K_m) was also calculated in unit of ml/min per mg protein.

2.5. Analytical methods

EN and NA concentrations were assayed by high performance liquid chromatography as in our previous report [11]. Briefly, a mobile phase consisting of methanol and 0.1% (v/v) of phosphoric acid (65:35, v/v), and methanol: 0.1% (v/v) (80:20, v/v) containing 5 mM of sodium 1-heptane sulfonate were used for EN and NA. The internal standards for EN and NA were methylparaben and *p*-hydroxybenzoic acid, respectively. It was confirmed that hydrolysis of EN to NA occurs only via the metabolic pathway [11,15].

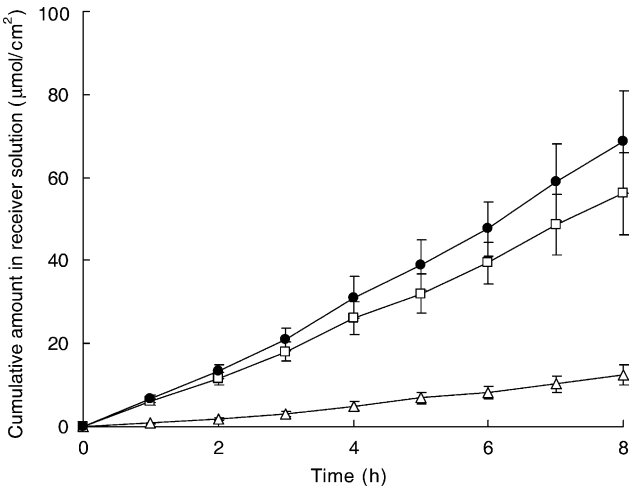

Fig. 1. Permeation profiles through human skin from EN-saturated solution (244 mM). □, EN; △, NA; ●, total. Each point represents the mean ± SD of three to four experiments.

2.6. Statistical analysis

Differences in flux of EN, NA and total, and NA flux/total flux ratio were statistically evaluated by one-way ANOVA following Dunnett's test.

3. Results

3.1. Skin transport study

In vitro skin permeation and metabolism of EN using rabbit, rat, guinea-pig, pig, shed snake skin and human was performed. Fig. 1 represents the skin permeation profile through human skin from an EN-saturated solution (244 mM). The cumulative amount of EN, NA and the total in the receiver chamber increased linearly with time after a short lag time (about 20 min). This linear increase was also observed in other profiles for EN concentrations and for species (data not shown).

The flux of EN, NA and the total in the steady-state of EN-saturated solution are shown in Table 1. EN flux through pig, human and shed snake skin was significantly higher than that through rabbit, rat and guinea-pig skin, whereas NA flux through rabbit, rat and guinea-pig skin was significantly higher than that through human and pig skin ($P < 0.05$). Total flux of rat, guinea-pig and rabbit was significantly higher than that of human ($P < 0.05$). There was no significant difference in the total fluxes between pig skin, shed snake skin and human skin ($P > 0.05$). The ratio of NA flux to total flux ratio showed a similar tendency of NA flux.

The effects of donor concentration of EN on the flux of EN, NA and the total for various species are shown in Fig. 2. In proportion to donor concentration for all species, there is no direct relation between EN concentration and EN, NA and total flux. However, the greatest variation was found in NA flux. In rat, rabbit and guinea-pig, a large conversion to NA flux was observed throughout the donor concentration range (6–244 mM). For pig, shed snake, and human skin, EN flux gradually increased in the lower donor concentration (6–50 mM), and then sharply increased in the higher concentration (50–244 mM). On the other hand, NA flux sharply increased in the lower concentration and reached a plateau in higher concentration. The plateau values of NA flux for pig, snake and human were 1.95, 0.41 and 1.86 μmol/cm^2 per hour, while the donor concentration, in which the NA flux reached a plateau was around 50, 50 and 100 mM, respectively.

3.2. Skin homogenate hydrolysis study

Fig. 3 shows the Lineweaver–Burk plot between the inverse of hydrolysis rate and the inverse of EN concentration in skin homogenate of various species.

Table 1
Flux of EN and NA from saturated solution of EN through skin of various species

Species	Flux (μmol/cm^2 per hour)			NA/total flux ratio
	EN	NA	Total[a]	
Rabbit[b]	2.21 ± 0.06*	13.1 ± 0.8*	15.3 ± 0.9*	0.856 ± 0.032*
Rat[c]	2.68 ± 0.08*	11.2 ± 0.8*	13.8 ± 0.9*	0.812 ± 0.042*
Guinea-pig[d]	2.17 ± 0.09*	12.3 ± 0.7*	14.5 ± 0.8*	0.851 ± 0.028*
Pig[e]	7.84 ± 0.84	1.92 ± 0.34	9.76 ± 1.15	0.197 ± 0.067
Snake[f]	9.95 ± 0.91	0.392 ± 0.022*	10.3 ± 0.9	0.0381 ± 0.0055*
Human[g]	6.98 ± 1.43	1.84 ± 0.22	8.82 ± 1.44	0.209 ± 0.081

Each value represents the mean ± SD of three to four experiments. *$P < 0.05$ compared with human.
[a] Total = EN + NA.
[b] $n = 10$ from five rabbits.
[c] $n = 12$ from six rats.
[d] $n = 9$ from six guinea-pigs.
[e] $n = 10$ from five pigs.
[f] $n = 14$ from six snakes.
[g] $n = 8$ from five specimens.

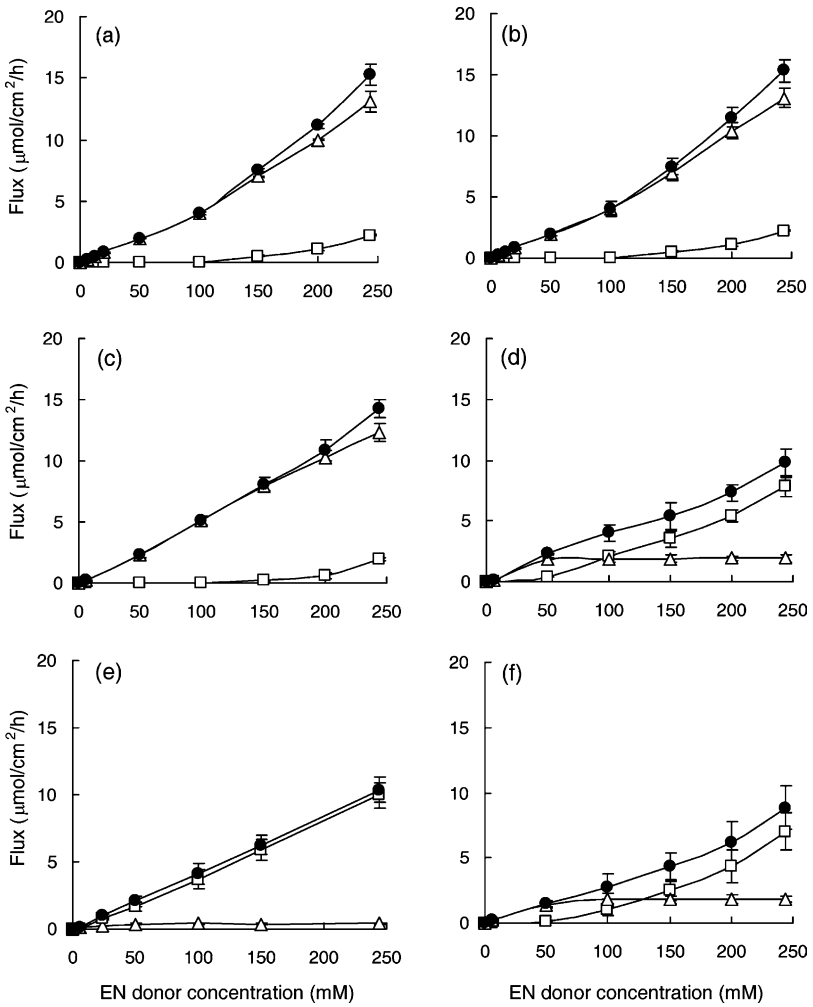

Fig. 2. Effect of EN concentration in the donor side on fluxes of EN, NA and total for rabbit (a), rat (b), guinea-pig (c), pig (d), shed snake skin (e), and human (f). □, EN; △, NA; ●, total. Each point represents the mean ± SD of three to four experiments.

Hydrolysis of EN to NA did not occur in PBS, but started in skin homogenate. The Michaelis–Menten process was observed in this enzymatic hydrolysis, therefore the kinetic parameters of EN in skin homogenate of various species were evaluated (Table 2). The maximum hydrolysis rate (V_{max}) of human skin was significantly lower than that of rabbit, rat and guinea-pig, but higher than shed snake skin ($P < 0.05$). The Michaelis–Menten constants (K_m) and intrinsic metabolic clearance (V_{max}/K_m) of human skin was significantly different compared with other species.

4. Discussion

In the present study, the skin permeation profile of EN was affected by the species difference. Both EN and NA were found in receiver solutions for all species in the skin permeation experiment (Fig. 1 and Table 1). Leakage of dermal enzyme from skin specimens into the receiver solution was not detected as discussed previously [11]. This finding implies that the conversion of EN to NA occurs in the skin during the transdermal absorption process. The activity of skin esterase during the experimental period was observed to be constant during the experimental period due to linearity of the increase in the cumulative amount of NA with time (Fig. 1). In this study, the whole skin (epidermis and dermis) of different species was used except shed snake skin. The metabolic capacity in the cell, responsible for metabolism in skin layers, namely keratinocytes in epidermis and fibroblasts in dermis may differ between species. However, the higher esterase activity in the keratinocytes than that of fibroblasts has been reported in various compounds and species [16]. Therefore, variation in metabolic capacity in different species might be from the different activity in keratinocytes.

EN permeated across the stratum corneum as an intact form and was in some degree hydrolyzed to NA by esterases in viable skin, then both EN and NA concomitantly diffused into the receiver solution. Thus, the flux of EN through the stratum corneum is equal to the sum of EN and NA fluxes, namely the total flux through intact skin [9,11]. Theoretically, the total flux-conventional profiles should

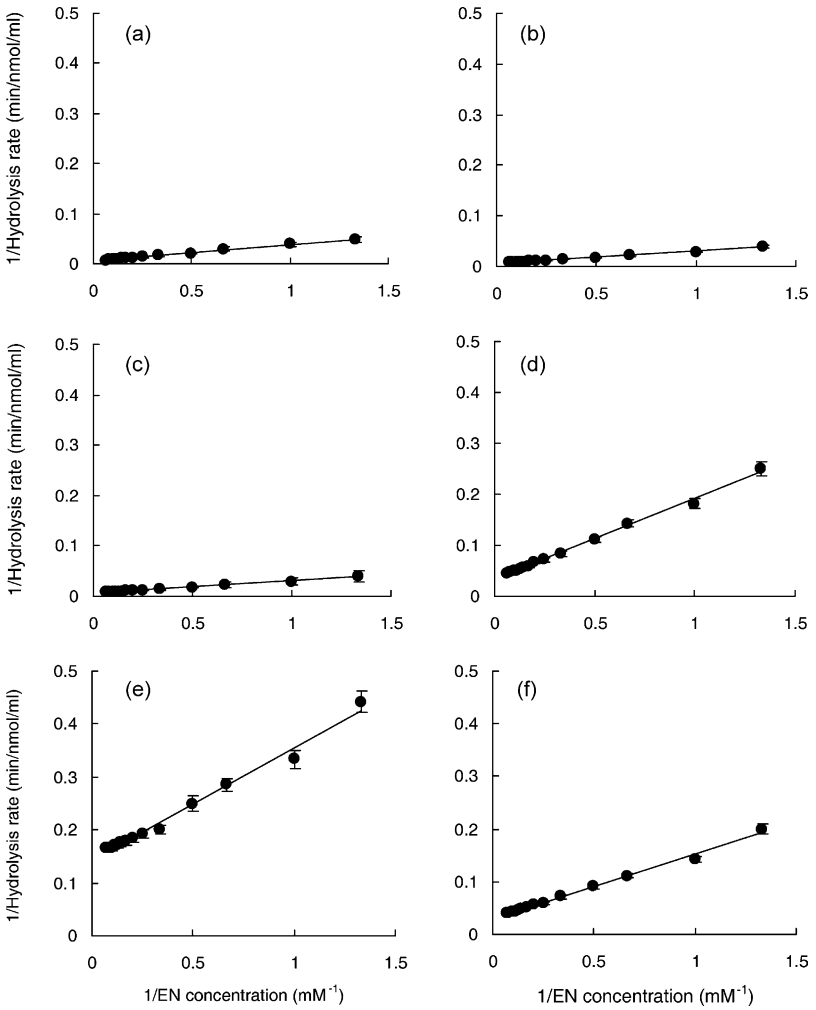

Fig. 3. Relationship between initial concentration of EN and hydrolysis rate in skin homogenate for rabbit (a), rat (b), guinea-pig (c), pig (d), shed snake skin (e), and human (f). □, EN; △, NA; ●, total. Each point represents the mean ± SD of three to five experiments.

be linear as Fick's first law of diffusion. However, the nonlinearity in those profiles can be explained that EN in higher concentration may effect on stratum corneum permeability. These data were similar to the penetration flux of phenol through rat skin [17]. The total flux of rat, guinea-pig and rabbit was significantly higher than that of human (Table 1, $P < 0.05$). However, the similarity of the total fluxes between pig, shed snake and human skin was observed. The total flux of rat, guinea-pig and rabbit was significantly higher than that of human (Table 1, $P < 0.05$). Norggard [18] reported that the skin permeability rates for cobalt ions was rabbit > guinea-pig > human. The difference in the skin permeability among different species might be caused from the divergence in lipid content and water uptake of the stratum corneum, epidermal and dermal thickness and density of hair follicles in the skin [18–20]. However, the similarity of the total fluxes between pig, shed snake and human skin was observed (Table 1). This might be due to the similarity in thickness and lipid content of the stratum corneum (17.5 ± 2.4 μm, 4–8% (pig);

Table 2
Michaelis–Menten parameters of EN in hydrolysis experiment using skin of various species

Species	V_{max} (nmol/min per mg protein)	K_m (mM)	V_{max}/K_m (ml/min per mg protein)
Rabbit	170.5 ± 7.2*	6.28 ± 0.48*	0.0347 ± 0.0073*
Rat	202.5 ± 6.5*	5.84 ± 0.45*	0.0568 ± 0.0092*
Guinea-pig	181.2 ± 5.5*	3.19 ± 0.34*	0.0271 ± 0.0053*
Pig	29.3 ± 2.1	2.21 ± 0.32*	0.0133 ± 0.0009*
Snake	6.56 ± 0.9*	3.58 ± 0.21*	0.00183 ± 0.00082*
Human	32.5 ± 1.9	1.01 ± 0.09	0.0322 ± 0.0007

Each value represents the mean ± SD of three to four experiments. *$P < 0.05$ compared with human.

16.4 ± 3.4 μm, 6% (shed snake); 18.2 ± 3.3 μm, 2–6.5% (human)) [21,22].

Compared with skin permeability, dermal metabolism in different species was considerably varied. NA flux, NA to total flux ratio, V_{max}, and V_{max}/K_m was about 22-fold different between the maximum of rabbit and the minimum of snake. NA flux, NA to total flux ratio, V_{max}, and V_{max}/K_m of human was significantly lower than those of rabbit, rat and guinea-pig, but higher than those of shed snake skin. There was no significant difference between these parameters of human and pig. These findings indicated that the total esterase content in human skin is different from the other species tested except pig skin. Species difference in skin metabolism of benzo(a)pyrene and testosterone in six mammalian species including humans was reported, and both rabbit and guinea-pig skin have a higher level of enzyme activity than human skin [7]. Rat skin has a higher level of esterase activity (20 times) than human skin for the metabolism of 6-α-methylprednisolone-17-propionate-21-acetate [23]. The higher metabolism in human than the shed snake skin might be due to the difference in esterase contents in the skin layer. Human skin is composed of stratum corneum and viable skin (epidermis and dermis), whereas shed snake skin has only a horny layer. In the mass of dead cells of the stratum corneum of shed snake skin, esterase enzymes are found to be active [8]. However, a major part of skin metabolic activity is located within the viable epidermis layer [24]. Thus, the total esterase content in the human skin is expected to be higher than that in the shed snake skin.

In case where the EN concentration (C) in the skin is significantly lower than K_m, the metabolic clearance $[V_{max}/(K_m + C)]$ is almost equal to V_{max}/K_m. In the lower concentration range, EN permeation through the stratum corneum is to be the rate-limiting step in the NA permeation process. On the other hand, in the higher concentration range, metabolic clearance was reduced, and the rate-limiting step is to change from permeation to metabolism of EN. Especially in the metabolic saturation condition, NA flux must be the same as V_{max}, theoretically. In our experiment, metabolic saturation was taking place at a higher concentration range of EN shed snake, pig and human skin. In rabbit, rat and guinea-pig, contribution of NA to total flux was decreased in EN-saturated solution, suggesting that metabolism of EN to NA was close to saturation. In order to compare NA flux in the skin permeation study with V_{max} in the skin homogenate hydrolysis experiment, V_{max} in unit of μmol/cm^2 per hour was utilized. The recalculated V_{max} of pig, snake and human skin (1.45, 0.24, 1.33 μmol/cm^2 per hour, respectively) was consistent with the NA flux at the plateau region in each species, whereas that of rabbit, rat and guinea-pig was much higher (11.5, 10.7, 11.1 μmol/cm^2 per hour). These might be discussed with the difference in extent of skin esterase enzyme in the skin and in the skin enzyme distribution [25,26].

The skin permeation data and K_m could not be simply compared because the real EN concentration in the skin could not be estimated. The donor concentration of EN, in which the NA flux reached a plateau was about 50 mM for snake, 50 mM for pig, and 100 mM for human (Fig. 2). K_m values of each species were varied (Table 2), suggesting that the affinity of esterases for EN in each species is different. If skin structure was similar among species, EN concentration in the skin should be proportional to that in the donor solution, and thus the donor concentration in which the NA flux reached a plateau should be independent of species. However, there are some structural differences in the skin among species, such as the difference in the hair follicles and surface lipid content [19,21]. Therefore, EN concentration in the skin could be expected to be different in each species after applying the same concentration of EN in the donor side.

5. Conclusion

The present study verified the difference in skin permeation and dermal esterase activities in different species. The discrepancy in permeation profiles of EN among the species tested was mainly due to the difference in esterase activity. To predict the skin permeability and metabolism of human skin by extrapolating from the data on animal skin, differences in skin metabolism should be taken into consideration. In our study, permeability profile and esterase activity of pig skin was similar to human skin. However, in vivo study using other permeants should be tested to obtain more data.

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