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Research paper

Comparison of skin distribution of hydrolytic activity for bioconversion of β-estradiol 17-acetate between man and several animals in vitro

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Abstract

We have investigated the distribution of hydrolytic enzymes which metabolize β-estradiol 17-acetate (EA) to β-estradiol (E) in man and animal skins in vitro. The distribution of hydrolytic enzymes in human cadaver, hairless dog, rat and hairless mouse skin, was investigated by a skin-slicing technique. We performed histological studies with hematoxylin and eosin stain. The highest amount of metabolite (E) appeared in the layers of 80–120 μm from the skin surface, the basement layer in human skin, while the amount of metabolite was distributed evenly in the hairless dog skin from 0 to 180 μm. In the rat and hairless mouse skin, on the other hand, peak levels of metabolite were observed in the basement layer of dermis, the surrounding area of the cutaneous plexus. The total metabolic activities in the area of epidermis in human, hairless dog and hairless mouse skin were 2.59, 8.03 and 0.33×10^{-4} μg/ml/μm/h, respectively. The values in whole skin layers in the hairless dog and hairless mouse skin were 3.35 and 1.85×10^{-4} μg/ml/μm/h, respectively. EA transported across the human and hairless dog skin can be effectively metabolized before entering the capillary. Among animal models investigated, hairless dog skin might be the most facile model in simulating drug metabolism for human skin under the clinical (in vivo) conditions. Hairless mouse skin, on the other hand, was also an excellent model in excised human skin under in vitro conditions.

Keywords: Skin distribution of metabolite; Hydrolytic enzyme; β-Estradiol 17-acetate; β-Estradiol; Human skin; Hairless dog skin; Rat skin; Hairless mouse skin

1. Introduction

Skin metabolism or permeability of drugs differ among animal species; skin penetration of selegiline, an inhibitor of monoamine oxidase-B (MAO-B), in humans was similar to that in dogs but significantly differed from hairless mice, rats and Micropig® [1]. Biodegradation rates of stereoisomers of soman, an organophosphorus compound, in skin homogenate differed among guinea-pig, mouse, human and hairless mouse [2]. Only the hairless mouse skin had binding sites for soman isomer [2]. Dinitrochlorobenzene (DNCB) absorption through mouse, rat, pig and human skin in vitro and skin metabolism by glutathione transferase were determined [3]. Absorption and glutathione conjugation formation in pig skin were similar to that in human skin [3].

The skin distribution of hydrolytic activity which metabolized β-estradiol 17-acetate (EA) to β-estradiol (E) differs among animal species [4]. Hydrolytic activity for bioconversion of prednisolone 21-acetate to prednisolone was distributed in the epidermis of human skin, especially in the basement membrane [5]. This finding suggests that the distribution of hydrolytic activity in human skin may prevent certain substances from entering the systemic circulation in their unhydrolyzed form. Distribution of the enzymatic activity in human and animal skin should be understood to develop reliable in vivo/in vitro and human/animal relationships as well as to optimize transdermal drug delivery systems.

This paper compares the hydrolytic activity in the skin for metabolism of EA to E among human cadaver, hairless dog, rat, and hairless mouse skins in vitro by a skin slicing technique [6]. We performed cutaneous histological studies to

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interpret the distribution of the enzymatic activity among animal species.

2. Materials and methods

2.1. Materials

β-Estradiol 17-acetate (EA, reagent grade, minimum 99%) and β-estradiol (E, reagent grade, minimum 98%) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Polyethylene glycol 400 (PEG 400), acetonitrile (HPLC grade), and distilled water (D.W., HPLC grade) were purchased from Wako Pure Chemical Co. Ltd. (Osaka, Japan).

Human cadaver skin samples were removed from four Caucasian males (age 41, 48, 71 and 73 years, back and leg skin). The skin samples remained in Eagle minimum essential medium at 4°C until use. Storing the skin samples at 4°C caused enzymatic deactivation [7], but we did not regard it as important because we examined the skin distribution of metabolite and not the enzymatic kinetic parameters, as a first rate constant. Animal model skin samples, hairless dog (female, 7–9 years old), rat (Wister:-Kud, female, 8–10 weeks old) and hairless mouse (Hr-/Kud, female, 8–10 weeks old), were obtained from Nihon Nosan Kogyo Co. (Tsukuba, Ibaraki, Japan) and Kyudo Co. (Tosu, Saga, Japan), respectively. Hairless dog skin was obtained from male Mexican hairless dogs, generally referred to as Xoloitzcuintli in Mexico, and female beagle at Nihon Nosan Kogyo, Co. Hairless dog skin was reported to be a useful model for investigation of photodermatology and the treatment of comedones, pigmentation and alopecia [8]; skin permeability of some drugs was also compared with that of man [9,10]. All animal studies conformed to the 'Principles of Laboratory Animal Care' (NIH publication #85-23, revised 1985).

2.2. Methods

2.2.1. Distribution of drugs metabolized in the skin

After being frozen in TISSUE-TEK® optimum cutting temperature (OCT) compound (10.24% polyvinyl alcohol and 4.26% polyethylene glycol, Sakura Finetek USA, Inc., Torrance, CA, USA) at −20°C, the skin was sliced repeatedly from the surface into 10 μm thick sections with a microtome (LEICA CM 1850, Leica Microsystems Inc., Deerfield, IL, USA). Four or six consecutively sliced skins were placed into 5 ml of 40% PEG400 solution with a 60–80% concentration of EA saturated concentration (26.9 ± 1.0 μg/ml). The slices were then incubated in the elution medium at 36°C. Samples (750 μl) of the solution were withdrawn at predetermined time intervals and the same volume of ethanol was added to the sample for removing proteins and the OCT compound. The mixture of 40% PEG400 solution and ethanol was then centrifuged for 10 min at 10,000 × *g*. After filtering the supernatant (0.45 μm PTFE, Alltech Association Inc., Deerfield, IL, USA), the concentrations of EA and E were assayed by high performance liquid chromatography (HPLC). We previously reported that hydrolysis activity was inhibited by skin dehydration in the presence of PEG400 [11]. In order to investigate the spatial distribution rather than the intrinsic enzymatic kinetics of EA hydrolysis, we used 40% PEG400 solution for increasing the solubility of EA and E in this study.

2.2.2. Histological examination of the skin

Skin specimens were dehydrated by ethanol and xylene and then embedded in paraffin. Paraffin sections (4 or 6 μm) were hematoxylin and eosin stained and observed using a CCD camera (CCD-f2 MICRO CCD SCOPE, Shimadzu Co. Ltd., Kyoto, Japan).

2.2.3. Assay methods

EA and E were assayed by a modified HPLC (LC 10A, Shimadzu Co., Kyoto, Japan) method [4]; the column was YMC-Pack ODS-AM (250 × 4.6 mm^2 ID, S-5 μm, 120A, YMC Co. Ltd., Tokyo, Japan) with guard column (TSK-Gel 80Ts, Tosoh, Tokyo, Japan). The detector was operated at a wavelength of 205 nm. The mobile phase was a mixture of acetonitrile and distilled water. The acetonitrile fraction was gradually varied from 45 to 80% in order to separate E and EA. A flow rate of 1.0 ml/min resulted in a retention time of 10 and 23 min for E and EA, respectively.

3. Results

3.1. Skin distribution of hydrolytic activity of EA

To determine the skin distribution of hydrolytic activity of EA bioconversion, the slices microtomed from the skin were placed into EA solution. This skin-slicing technique represents the hydrolytic activity in the skin [4,5]. Fig. 1 shows the distribution and the time variation of metabolite E concentration in human skin. The amount of metabolite at each skin layer had an individual difference but the pattern of skin distribution, which showed a peak value at a distance of 80–120 μm from the skin surface, agreed well with human skin irrespective of age (41 (a), 48 (b), and 71 (c) years old). In Fig. 1c, a significant increase of metabolite E concentration was observed in the upper layers (40–200 μm), but not in the deeper layers (360–560 μm) between 24 and 48 h. This phenomenon was caused by the total amount of existing hydrolytic enzymes in each skin layers and deactivation of enzyme with time. Figs. 2–4 show the concentration of metabolite (E) in hairless dog, rat and hairless mouse skin, respectively. In the previous experiments of skin penetration/metabolism of EA, the flux of metabolite E across stripped hairless dog skin, in which stratum corneum was completely removed by tape-stripping, decreased with time over 18 h, indicating skin

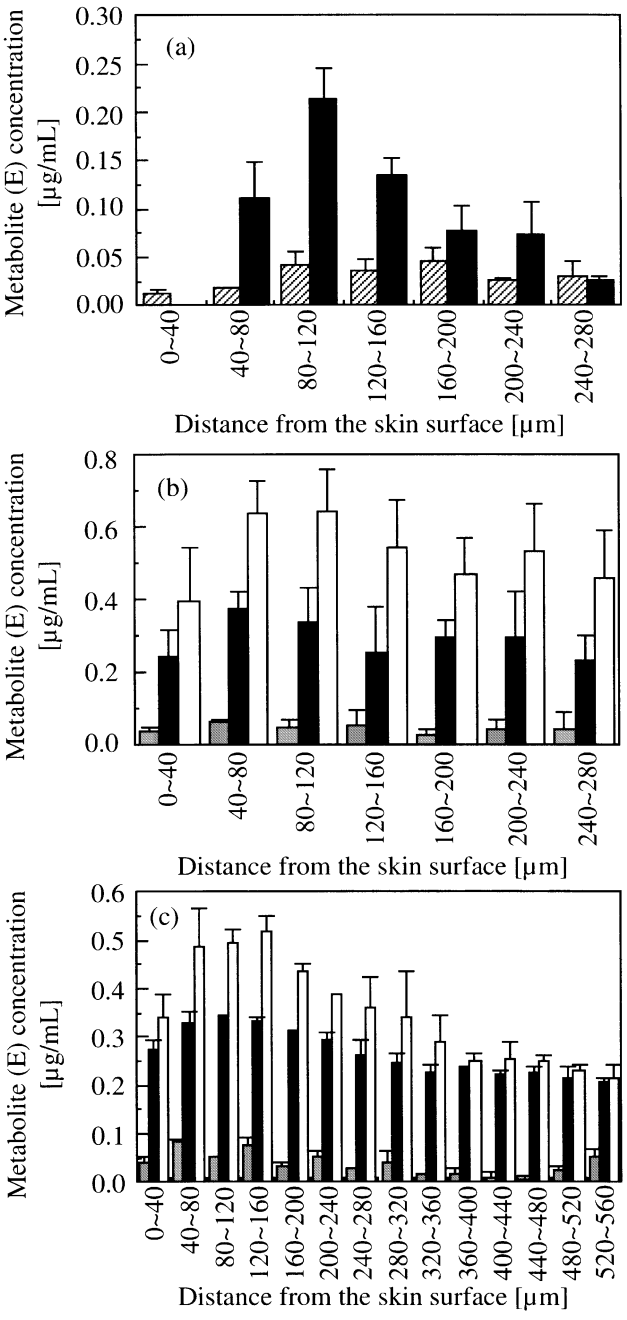

Fig. 1. Distribution and time dependence of EA metabolite (E) concentration in the EA solution after 3 h (▨), 6 h (▩), 24 h (■) and 48 h (□). Skin samples were removed from 41 (a), 48 (b) and 71 (c) years old male Caucasian cadavers. The columns indicate mean ± AD (a and c, $n = 2$) and mean ± SD (b, $n = 4$).

enzymes deactivated with time [12]. On the other hand, the flux of metabolite across the stripped skin of rat and hairless mouse linearly increased over 24 h [12]. Therefore, we estimated the skin distribution pattern at 12 h for hairless dog and at 24 h for rat and hairless mouse. The amount of metabolite was constant from 0 to 180 μm in hairless dog skin after 12 h incubation. Rat and hairless mouse skin had the highest metabolic activity at 360–420 μm and 180–240 μm from the skin surface respectively after 24 h incubation.

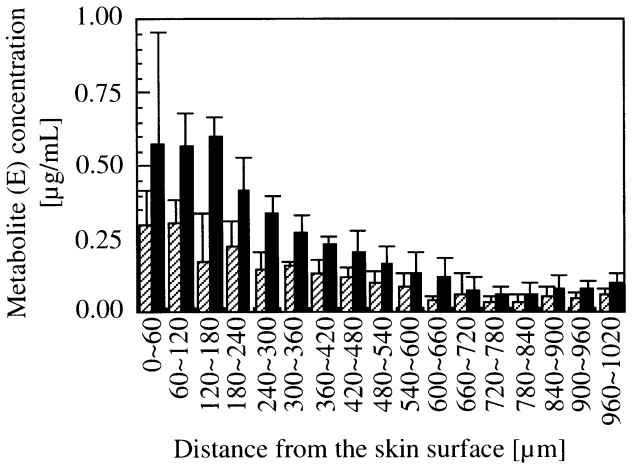

Fig. 2. Distribution and time dependence of metabolite (E) concentration in the EA solution after 3 h (▨) and 12 h (■). Samples were removed from dorsal skin of hairless dog (female, 7–9 years old). The columns indicate mean ± SD ($n = 3$).

3.2. Histology of the skin

We stained skin sections with hematoxylin and eosin to demonstrate the histological difference among human and animal skins. Fig. 5 shows the CCD camera image of a skin section of human (a), hairless dog (b), rat (c) and hairless mouse (d), respectively. Table 1 summarizes the thickness of epidermis and dermis estimated from CCD camera image. The thickness of human epidermis averaged 89 μm corresponding to the region where the maximum concentration of the metabolite was observed (Fig. 1). The thickness of hairless dog and hairless mouse was about 40 μm and the rat (about 30 μm) was the thinnest of three animal skins. On the other hand, the thickness of dermis of human, rat and hairless mouse averaged 260, 630 and 290 μm, respectively, and that of hairless dog was less than one-third of human dermis.

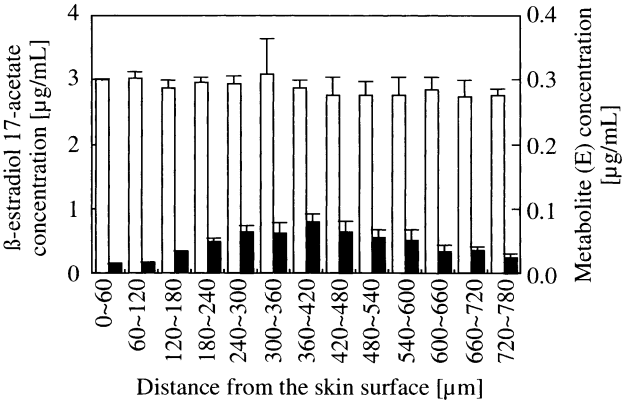

Fig. 3. Concentration profiles of EA (□) and its metabolite (E, ■) in the EA solution as a function of the distance from the skin surface after 24 h incubation. Skin samples were removed from rat (Kud:Wister, female, 8–10 weeks old). The columns indicate mean ± SD ($n = 3$).

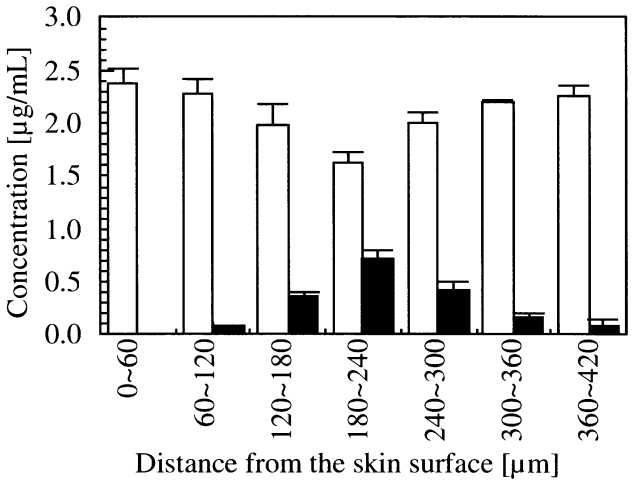

Fig. 4. Concentration profiles of EA (□) and its metabolite (E, ■) in the EA solution as a function of the distance from the skin surface after 24 h incubation. Skin samples were removed from hairless mouse (Hr-/Kud, female, 8–10 weeks old). The columns indicate mean ± SD ($n = 3$).

Table 1
The thickness of epidermis and dermis evaluated from CCD camera image

	Epidermis (μm)	Dermis (μm)
Human	38.8–138.4	243.2–287.2
Hairless dog	23.5–61.3	61.6–104.6
Rat	15.7–42.6	543.1–717.6
Hairless mouse	21.6–73.0	197.5–391.7

4. Discussion

In this study, the enzymatic activity responsible for EA metabolism in the human skin was mainly distributed at a distance of 80–120 μm from the skin surface (Fig. 1a–c). We previously reported that hydrolytic activity which metabolized prednisolone 21-acetate in human skin was distributed in the same layer [5]. The histological study indicated that the layer was the basement layer (Table 1) and the papillary plexus lies histologically just beneath this [13–15]. This distribution pattern indicates that the hydrolytic

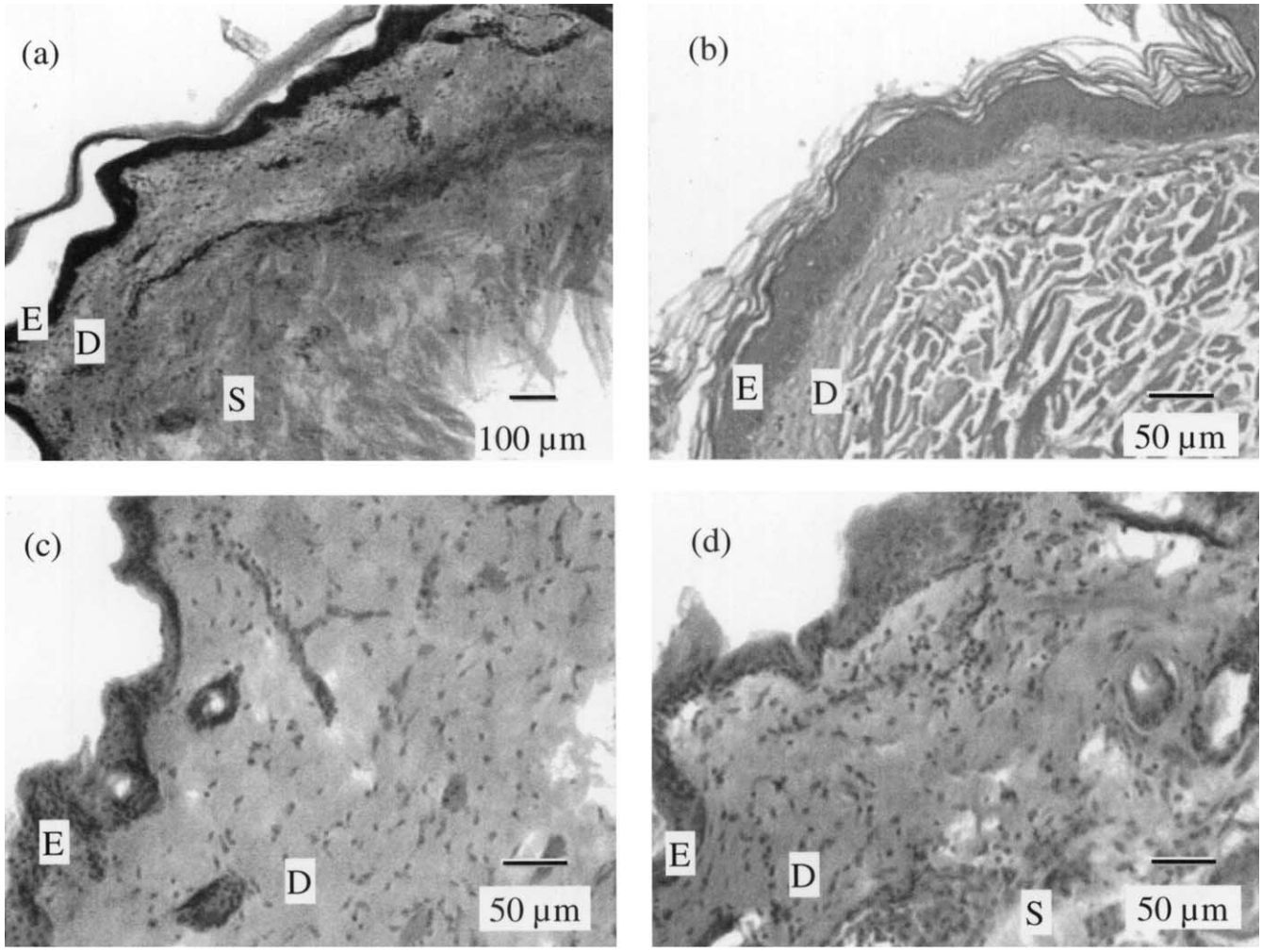

Fig. 5. CCD camera image of a skin section of human (a, Caucasian male, 73 years old), hairless dog (b, female, 6 years old), rat (c, Kud:Wister, female, 10 weeks old) and hairless mouse (d, Hr^{-}/Kud, female, 8 weeks old). Skin section was stained by hematoxylin and eosin. Magnification is ×210 for human skin and ×600 for hairless dog, rat and hairless mouse. The layers are epidermis (E), dermis (D) and subcutaneous layer (S).

enzymes in this layer may efficiently metabolize transdermally applied ester drugs to the parent drug before entering the systemic circulation. Hydrolytic activity in hairless dog skin was constant in the first 180 μm and then decreased with increasing distance from the skin surface (Fig. 2), although the thickness of epidermis was about 40 μm (Table 1). Thus, the hydrolytic activity in hairless dog skin exists uniformly in epidermis and also in the upper layer of the dermis.

On the other hand, rat and hairless mouse skin have the highest metabolic activity in the layer of 360–420 and 180–240 μm (Figs. 3 and 4) from the skin surface, respectively. We assume that rodents had the highest activity hydrolyzed EA at the surrounding area of the basement layer of dermis (Table 1). The dermal microvascular network consists of two systems; one in the above-mentioned papillary plexus and the other in the deep vascular plexus, the cutaneous plexus [16]. Thus, rodents may have the highest hydrolytic activity of the surrounding area of the cutaneous plexus in contrast to human and hairless dog skin. The cutaneous plexus is connected by arterioles and venules to the papillary plexus and lies above the boundary between the dermis and subcutis [17]. The papillary plexus mainly transports the transdermally applied chemicals into the circulation. Therefore, rodents take topically applied drugs into the circulatory system from the papillary plexus before metabolizing and detoxifying them.

The distance of epidermis from the skin surface represents the distance of the capillary plexus. Thus, transdermally applied chemicals to human skin are bioconverted only by enzymes existing in epidermis and the metabolic activity in the skin under in vivo and in vitro experimental conditions may be estimated from the concentration of metabolite in the area of epidermis and whole skin, respectively (Fig. 1). Similarly, we expected that the amount entering the circulation to be estimated from the amount of metabolite in these layers. We, therefore, defined in vivo and in vitro conditions as the amount of metabolite per time and thickness in the area of epidermis and in the whole skin, respectively. Table 2 summarizes the estimated metabolic activity of human and several animal skins under in vivo and in vitro conditions. Although the value of hairless dog skin under in vivo condition (8.03×10^{-4} μg/ml/μm/h) was over-estimated in comparison with that of human skin (2.59×10^{-4} μg/ml/μm/h), hairless dog skin was closer to human skin than the other animals. On the other hand, hairless mouse (1.85×10^{-4} μg/ml/μm/h) and hairless dog (3.35×10^{-4} μg/ml/μm/h) skin under in vitro condition resembled human skin under in vivo conditions. This may indicate that hairless dog skin could be a good model to represent clinical metabolizing phenomena in human skin in vivo; the distribution of hydrolytic activity in hairless dog skin also resembled human skin. Under in vitro conditions, on the other hand, hairless mouse skin may be also an excellent model to simulate clinical conditions.

Table 2
Metabolic activity of EA through human and several animal skin under in vivo and in vitro conditions

	In vivo[a] ($\times 10^4$ μg/ml/μm/h)	In vitro[b] ($\times 10^4$ μg/ml/μm/h)
Human	2.59	
Hairless dog	8.03	3.35
Rat	0.12	0.34
Hairless mouse	0.33	1.85

[a] In vivo condition represents the amount of metabolite per thickness in the area of epidermis. Thickness of epidermis see Table 1.

[b] In vitro condition represents the amount of metabolite per thickness in the whole skin.

5. Conclusions

Human skin has the highest hydrolytic activity in the basement layer of epidermis, while hairless dog skin has uniform distribution in the epidermis. Rat and hairless mouse skin have peak enzymatic activity in the basement layer of the dermis. These findings indicate that human and hairless dog skins have a superior biological barrier compared to rat and hairless mouse skin under in vivo experimental condition. Hairless dog and hairless mouse skin may be a good model to simulate the clinical situation for hydrolytic activity which bioconvert EA to E under in vivo and in vitro experimental conditions, respectively.

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