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Research paper

Bioconversion of estradiol esters in the skin of various animal models in vitro

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Abstract

This study explored the distribution of the metabolic activity of estradiol esters in the skin of various animal models including hairless mice, mice, hairless rats, Wistar rats and rabbits. The concentration distribution of the estradiol prodrugs, β-estradiol 3,17 diacetate (ED), β-estradiol 17 acetate (EA) and its metabolite β-estradiol (E) in the thin skin layers sliced from the skin surface was measured in the elution medium containing the prodrugs after 3, 6, 12 and 24 h incubation. For EA to E metabolism, a peak concentration of estradiol appeared in the middle layers of the hairless mouse skin, suggesting that the enzymes responsible for EA to E metabolism are distributed most around the middle layers of the skin. Not only the distribution profile but the magnitude of the metabolic activity in the skin was influenced by the animal models used; the enzymatic activity of EA to E bioconversion in the hairless mouse and mouse skin was much greater than that in the rat, Wistar rat and rabbit skin. The skin metabolism of EA to E was completely inhibited by diisopropyl fluorophosphate (DFP) while ED to EA metabolism was hardly inhibited by DFP. © 1997 Elsevier Science B.V.

Keywords: Prodrug; Skin metabolism; Enzyme distribution; Estradiol; Estradiol esters; Transdermal delivery

1. Introduction

Pharmaceutical researchers have investigated the percutaneous absorption of a variety of compounds for developing topical formulations as well as systemic transdermal delivery systems [1–3]. Nevertheless, the details of percutaneous absorption accompanied by skin metabolism remains unclear for many drugs. For instance, the metabolic activity in the skin is widely assumed to be homogeneous in the viable skin for the simplification of analysis of experimental data obtained. The penetration of the drug and its metabolites is evidently influenced by the distribution of metabolic activity in the skin, it is therefore essential to develop a transdermal drug delivery system by prodrug bioconversion [4–8].

The skin enzymes responsible for the certain metabolism in question have been identified by a skin staining technique in the literature [9,10]. However, this method does not provide proper information on the enzyme distribution in the skin. We must develop a detailed quantitative approach to the measurement of the distribution of metabolic activity in the skin.

In the present study, we have developed an approach to the measurement of the distribution of the metabolic activity in the skin in vitro. The concentration distribution of both drugs and metabolites was measured in sliced skin layers. Hairless mouse, mouse, hairless rat, Wistar rat and albino rabbit were used as animal models. The metabolic activity in the skin was evaluated by measuring the concentration of the drug and

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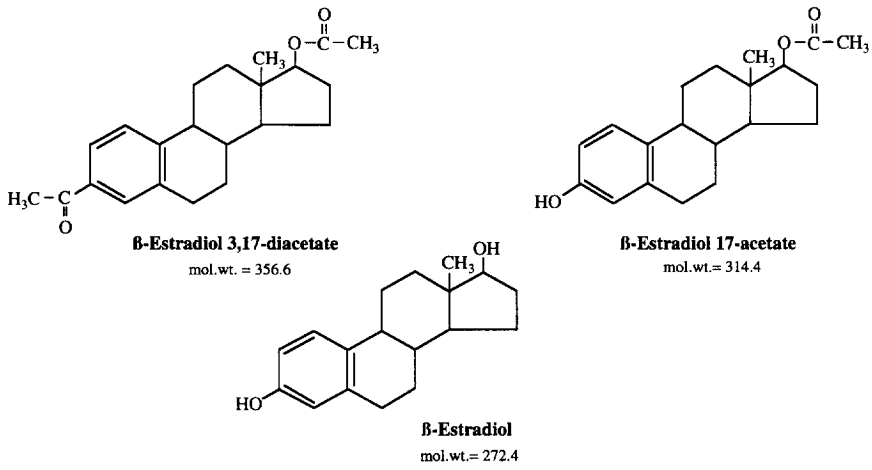

Fig. 1. Chemical sturcture of ED its metabolite, EA and E.

the metabolites in the skin layers sliced by a microtome after incubation. β-Estradiol 17 acetate (EA) and β-estradiol 3,17 diacetate (ED) were used as the model prodrugs.

2. Materials and methods

2.1. Materials

ED, EA and β-estradiol (E) (Fig. 1) were obtained from Sigma (St. Louis, MO). Polyethylene glycol 400 (PEG 400) was obtained from Kishida (Osaka, Japan). Acetonitrile and water as the HPLC mobile phase and diisopropyl fluorophosphate (DFP) were purchased from Wako Pure Chemical, (Osaka, Japan). Model animals, female hairless mice (Hr − /Kud, 8–10 weeks old), male mice (ddY/Kud, 8 weeks old), female hairless rats (Wistar hairless, 7 weeks old), female rats (Wistar, 7 weeks old) and Japanese albino rabbits (Jpo.w) were obtained from Kyudo (Tosu, Japan).

2.2. Measurement of the metabolic activity distribution

Cut to 2.2 × 1.8 cm, the intact dorsal skin was sliced into 21 pieces from the stratum corneum side by using the tissueteck microtome Cryostat (Milles Sankyo, Tokyo, Japan) at −20°C. Each sliced skin was controlled to be 20 μm thick. Every three pieces were incubated in 5 ml of 40% PEG 400 solution containing EA or ED for 3, 6, 12 and 24 h at 37°C. Samples of 300 μl were taken from the elution media at predetermined time intervals. Following addition of 100 μl ethanol and filtration with a membrane filter (pore size 0.45 μm), the samples were analyzed for prodrugs and estradiol concentrations by HPLC. ED, EA and E were stable during the entire period of incubation in the elution medium.

2.3. Penetration-bioconversion experiment

In the penetration-bioconversion experiment, the stripped skin was used; the intact dorsal skin of the hairless mouse was stripped 20 times with an adhesive tape (Nichiban LP-24, Nichiban Tokyo, Japan) to remove the stratum corneum completely. The thickness of the stripped skin was measured with a micrometer by sandwiching the skin specimen between two plastic sheets of known thickness. The stripped skin was then mounted in the side-by-side diffusion cell system [11]. A drug suspension in 40% PEG 400 solution was filled in the donor compartment, after the receptor compartment was filled with a fresh 40% PEG 400 solution. At predetermined time intervals, 100 μl samples were withdrawn from the receptor solution and then an equal volume of each solution was added, respectively. The experiment was carried out at 37°C.

2.4. Inhibition of metabolism

To specify the enzymes responsible for the skin metabolism of the estradiol esters, ED and EA, diisopropyl fluorophosphate (DFP) was used as an enzyme inhibitor. Intact or stripped hairless mouse skins were mounted between the side-by-side diffusion cells. The suspension of prodrug ED or EA with 1 mM DFP was then charged in the donor compartment after the receptor compartment was filled with a fresh 40% PEG 400 solution which contained 1 mM DFP as an enzyme inhibitor.

2.5. Assay procedure

ED, EA and E were assayed by HPLC (Pump module 126 and Detector module 166: Beckman Instrument, San Ramon, CA). The column was a reverse-phase μ-Bondapak C_{18}, 3.9 × 300 mm (Waters, Millford, MA). The UV detector was operated at a

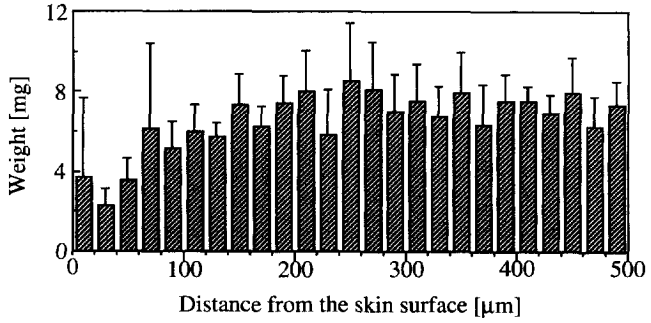

Fig. 2. Relationship between the weight distribution of sliced skin layers and the distance from the skin surface: Hairless mouse. Each value represents the mean ± S.D. of seven experiments.

wavelength of 205 nm [12]. The mobile phase was the mixture of acetonitrile and distilled water; the fraction of acetonitrile varied from 45 to 60% in order to separate E, and then 60–90% to separate EA and ED, completely. The flow rate of 1.0 ml/min resulted in a retention time of 9, 20 and 27 min for E, EA and ED, respectively.

3. Result and discussion

3.1. Distribution of metabolic activity

Fig. 2 shows the weight distribution of each sliced skin of the hairless mouse. Each skin layer was found to weigh approximately 7 mg except the surface three layers, suggesting that each microtome operation equally sliced the viable skin layer. Fig. 3 shows the concentration distribution of EA and E after a 6 h incubation. The concentration of EA decreased most in the middle layers of the skin, where the concentration of E reached the peak, correspondingly. This finding implies that the enzymes responsible for EA metabolism distribute most around the middle layers of

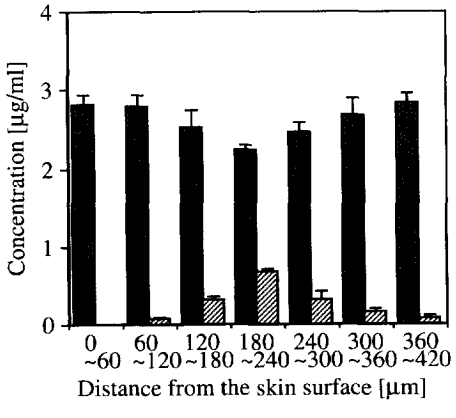

Fig. 3. Relationship between the concentration in the EA solution and the distance form the skin surface after 6 h incubation: Hairless mouse ($n = 3$). Initial EA concentration is 2.9 μg/ml. (dark shading), EA; (light shading), E.

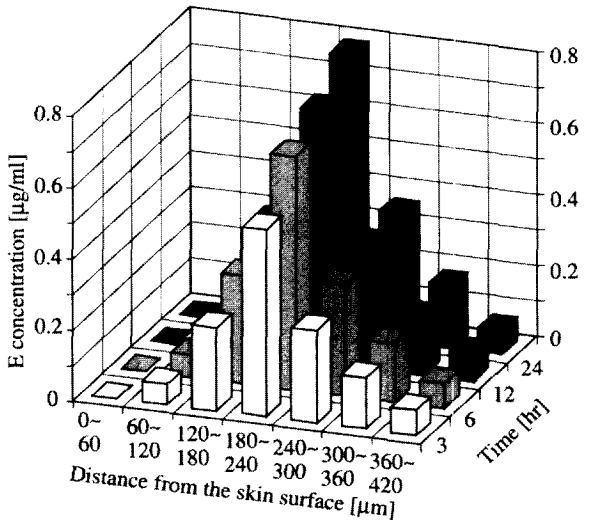

Fig. 4. Relationship between the concentration distribution of estradiol following the metabolism of EA and the distance form the skin surface: Hairless mouse.

the skin and negligibly near the surface including the stratum corneum. It is known that E may be metabolized to estron in the human skin [13]. In the hairless mouse skin, however, we detected no estron.

Fig. 4 shows the concentration distribution of E in the hairless mouse skin as a function of the incubation period. The concentration of E rapidly increased with increasing the period of incubation during the initial 6 (≈ 12) h. Beyond about 12 h, however, the concentration reached a plateau, possibly due to the saturation of metabolic reaction as well as the gradual deactivation of enzymes in the skin under the present in vitro incubation conditions. Tojo et al. previously found that the metabolic activity for the bioconversion of estradiol esters to estradiol in the hairless mouse skin decreased gradually after 6 h in the in vitro skin permeation experiment [14].

Interestingly, a unique pattern (mountain-shaped) of the distribution of E in the hairless mouse skin as shown in Fig. 4 was not influenced by the period of incubation (24 h).

Figs. 5–8 show the concentration distribution of EA and its metabolite, E, in the various animal skins: mouse, hairless rat, Wistar rat and rabbit, respectively. The concentration profile of EA or E in the mouse and hairless rat skin is similar to that in the hairless mouse; the highest metabolic activity appears in the middle layers of the skin. However, the estradiol concentration in Wistar rat skin homogeneously distributes in the viable skin (Fig. 7). In the rabbit skin, the E concentration was the highest near the surface of the skin and decreased gradually from the stratum corneum to the dermis side (Fig. 8).

Based on the ratio of concentration between the parent drug (E) and the prodrug (EA) (Figs. 5–8) not

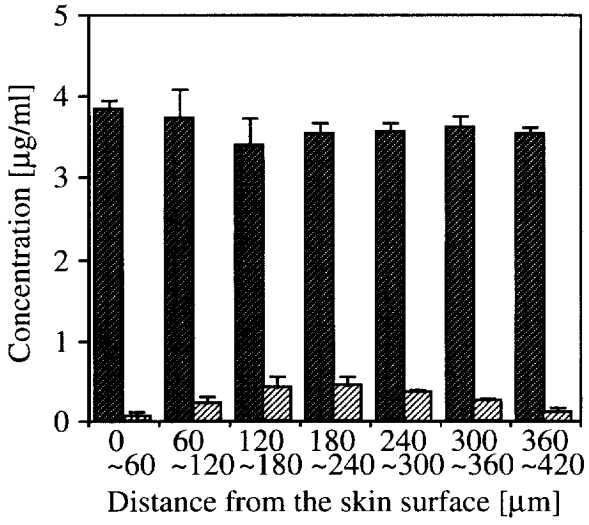

Fig. 5. Relationship between the concentration in the EA solution and the distance form the skin surface after 6 h incubation: Mouse ($n = 3$). Initial EA concentration is 4.5 μg/ml. (dark shading), EA; (light shading), E.

only the distribution profile but the magnitude of the enzymatic activity in the skin of mouse and hairless mouse is much greater than that in rat, Wistar rat and rabbit skins. Because of this low enzymatic activity in rat, Wistar rat and rabbit skins, the concentrations of E after incubation were within the error range of the detection of the equilibrium concentration of EA in Figs. 5–8. In Fig. 7, the equilibrium concentration of EA after incubation was almost equal to or slightly higher than the initial EA concentration. This occurred only in Wistar rat skin. At this stage of research, the reason for this finding is not clear. However, it can be concluded that the enzymatic activity of EA to E bioconversion distributes almost homogeneously throughout the skin of Wistar rat.

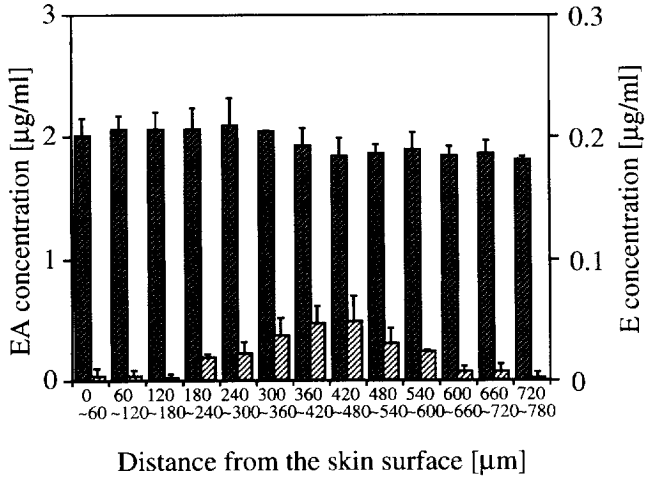

Fig. 6. Relationship between the concentration in the EA solution and the distance form the skin surface after 12 h incubation: Hairless rat ($n = 3$). Initial EA concentration is 2.1 μg/ml. (dark shading), EA; (light shading), E.

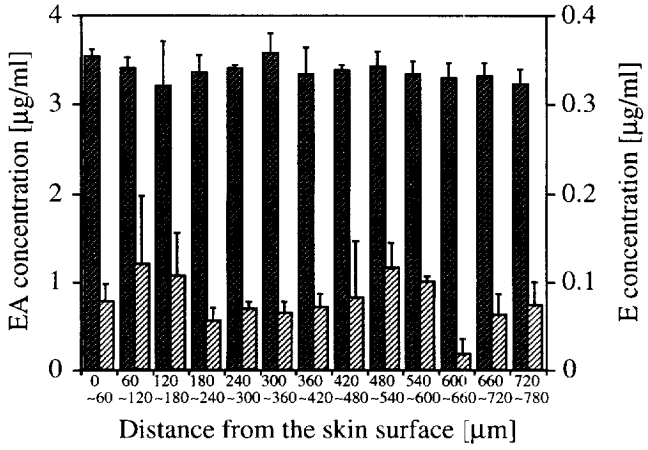

Fig. 7. Relationship between the concentration in the EA solution and the distance form the skin surface after 3 h incubation: Wistar rat ($n = 3$). Initial EA concentration is 3.2 μg/ml. (dark shading), EA; (light shading), E.

Fig. 9 shows the concentration distributions of ED, EA and E following skin metabolism of ED in the hairless mouse skin. The highest concentration of estradiol (E) was observed in the middle layer of the skin as was obtained for EA metabolism (Fig. 4). However, EA concentration distributes almost homogeneously throughout the skin layers, indicating that the enzyme responsible for the bioconversion of ED to EA may distribute evenly throughout the viable skin of the hairless mouse. As can be expected from the results obtained in EA to E bioconversion in the skin which varies between animal species, the homogeneous distribution of ED to EA bioconversion in the hairless mouse skin may not be applied to the other animal skins.

3.2. Inhibition

The effect of diisopropyl fluorophosphate (DFP) on the skin bioconversion of EA and ED was investigated in the hairless mouse skin.

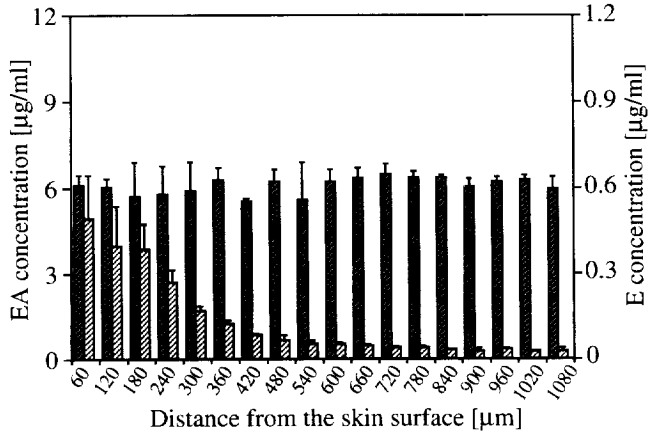

Fig. 8. Relationship between the concentration in the EA solution and the distance from the skin surface after 3 h incubation: Rabbit ($n = 30$). Initial EA concentration is 6.7 μg/ml. (dark shading), EA; (light shading), E.

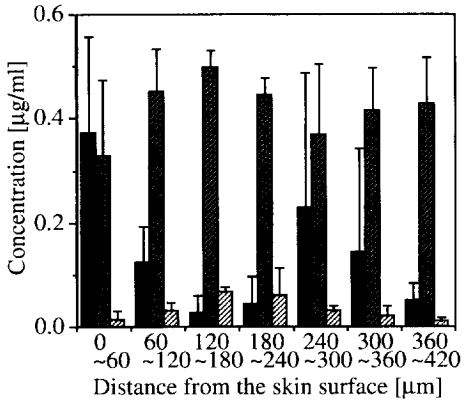

Fig. 9. Relationship between the concentration in the ED solution and the distance from the skin surface after 6 h incubation: Hairless mouse ($n = 3$). Initial ED concentration is 0.83 μg/ml. (dotted area), ED; (dark shading), EA; (light shading), E.

Fig. 10 shows the time courses of the cumulative amount of EA and E appearance in the receptor solution when DFP was added in the donor and receptor solution as a metabolism inhibitor [15]. It is clear that DFP completely inhibited the skin metabolism of EA to E. Fig. 11 shows the time courses of the cumulative amount of ED, EA and E appearances in the receptor solution following skin bioconversion of ED when DFP was added in the donor and receptor solution as a possible metabolism inhibitor. DFP evidently inhibits the bioconversion of EA but does not inhibit the metabolism of ED to EA. This finding clearly indicates that the enzymes responsible to ED to EA bioconversion differ from those for EA to E bioconversion.

4. Conclusion

A skin-sliced in vitro method has been developed to estimate the distribution of enzymatic activity for skin

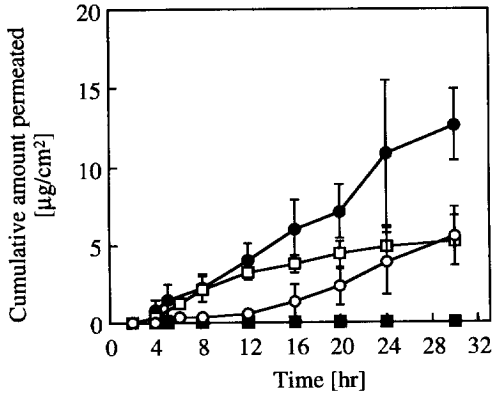

Fig. 10. Effect of DFP (1 mM) on time course of EA and its metabolite (E) across the hairless mouse stripped skin. ($n = 3$) ●, ED with DRP; ■, E with DRP; ○, EA without DFP; □, EA without DFP.

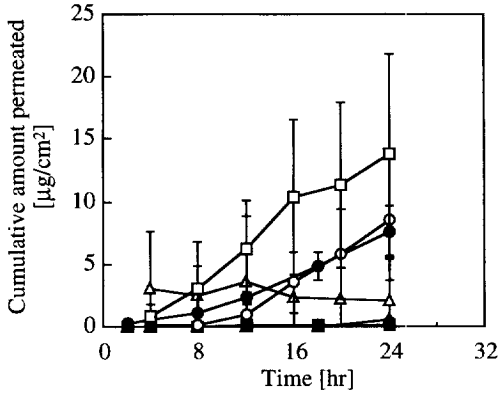

Fig. 11. Effect of DFP (1 mM) on time course of ED and its metabolite (EA, E) across the hairless mouse intact skin ($n = 3$). ▲, ED with DFP; ●, EA with DFP; ■, E with DFP; △, ED without DFP; ○, EA without DFP; □, E without DFP.

metabolism of estradiol esters. The enzymatic activity for EA was influenced by the animal species and is non-uniform in its distribution in the viable skin. For hairless mouse skin for instance, the highest activity for the metabolism was observed in the middle layers in the viable skin. While the enzymes responsible to the ED to EA bioconversion distribute almost homogeneously throughout the viable skin of the hairless mouse. DFP strongly inhibited the EA to E metabolism, and hardly inhibited the ED to EA metabolism, suggesting that the different esterases may be responsible for ED to EA and EA to E bioconversion in the hairless mouse skin. The understanding of drug transport mechanism across the skin accompanied by metabolism in the viable skin is essential for developing a transdermal drug delivery system by prodrug bioconversion. The distribution of the enzymatic activity and the inhibition discussed in the present study may be useful in developing a reliable in vivo/in vitro relationship as well as to optimize the transdermal prodrug delivery system.

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