Disparity of *in Vitro* and *in Vivo* Oleic Acid-Enhanced β-Estradiol Percutaneous Absorption Across Human Skin

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The permeation enhancing property of 5% oleic acid in ethanol on β-estradiol was investigated *in vitro* and *in vivo* using symmetrical and asymmetrical side-by-side diffusion cells and the human skin sandwich flap, respectively. β-Estradiol permeability *in vitro* and *in vivo* was similar in 75% ethanol (ETOH). Oleic acid (5%) did not alter β-estradiol permeability *in vivo* but increased permeability sixfold *in vitro* in symmetrical diffusion cells. β-Estradiol permeability in oleic acid was not different from that in ETOH, however, using asymmetrical diffusion cells. Stratum corneum-to-vehicle partition coefficients of β-estradiol in the vehicles were similar, yet fourfold more steroid was detected in skin biopsies from the *in vitro* symmetrical diffusion cells. Thus, oleic acid increased β-estradiol permeability *in vitro* only when skin was equilibrated with fatty acid. Attention to *in vitro* diffusion cell design and its relevance *in vivo* is critical to defining the mechanisms of enhanced solute permeation.

KEY WORDS: transdermal; human; *in vitro; in vivo;* estradiol; oleic acid; fatty acid; permeation enhancer.

INTRODUCTION

β-Estradiol permeation across the skin has been studied in a variety of delivery vehicles and experimental apparati (1–6). The addition of fatty acids to organic vehicles to enhance drug permeation is of interest in topical and transdermal drug delivery (7–11). Among those fatty acids investigated, oleic acid ($C_{18}H_{34}O_2$; molecular weight 282.5) has received the most interest. Oleic acid in propylene glycol has been demonstrated *in vitro* to enhance acyclovir permeation across human skin (7); in acetone, to enhance the permeation of mannitol, hydrocortisone, and progesterone across human skin (8); and in isopropanol, to enhance the permeation of cyclosporine A across rat skin (10). *In vitro* and *in vivo* biophysical analysis of human skin treated with oleic acid in alcohol, using Fourier transformed infrared spectroscopy, has demonstrated partitioning of the fatty acid into porcine (11) and human stratum corneum *in vivo* in a concentration-dependent manner up to 1% (12). Oleic acid uptake into stratum corneum has been associated with increased disorder of the stratum corneum lipids and thus, speculated, to be the mechanism of the increased permeation of coapplied drugs (11,12).

While many studies demonstrating oleic acid-enhanced permeation have been conducted *in vitro* using propylene glycol, there is a paucity of data on the influence of oleic acid on *in vivo* permeation of coapplied solutes in human skin in other organic vehicles. The present study evaluates the influence of 5% oleic acid in an ethanol vehicle on the permeation of the gonadal steroid, β-estradiol, across human skin *in vitro* and *in vivo*. *In vitro* permeation was measured using fresh split-thickness human abdominal skin mounted in side-by-side diffusion cells. *In vivo* percutaneous absorption of the fatty acid and steroid was measured using the grafted human skin sandwich flap model (13). This unique *in vivo* human skin model system has an isolated, yet accessible artery and vein that allow quantification of transdermal solutes in the local and systemic blood circulation. The ability to measure directly flux of the topically applied drug and drug disposition in the various skin layers of the treated skin over time in this *in vivo* model system enables a more detailed mechanistic evaluation of the percutaneous absorption process. Both human skin model systems were utilized to elucidate the physicochemical mechanisms by which such an enhanced permeation of transdermal β-estradiol with oleic acid might occur.

MATERIALS AND METHODS

[6,7-^{3}H(N)]17β-estradiol (sp act, 60 Ci/mmol) with 98% purity and [1-^{14}C]oleic acid (sp act, 58 mCi/mmol) with 99% purity were purchased from New England Nuclear Research Products, Dupont, Boston, MA. Crystalline β-estradiol and oleic acid were purchased from Sigma (St. Louis, MO). Oleic acid was stored under nitrogen at 4°C. Phosphate-buffered saline, pH 7.2, was prepared in the laboratory with potassium monobasic phosphate and sodium dibasic phosphate purchased from Sigma. In-house distilled water was used to prepare aqueous solutions. Ethanol (Gold Label; 95 parts ethanol, 5 parts isopropanol) was used as purchased from Aldrich (Milwaukee, WI). Trypsin No. T-1034 Type IX (Sigma), pH 8.0, was used to separate stratum corneum from split-thickness skin (14).

β-Estradiol solutions were prepared by adding excess drug to the 75/25 (v/v) ethanol:PBS (75% EtOH) or 5% oleic acid in 75/25 (v/v) ethanol:PBS (5% oleic acid) vehicles with unlabeled drug and allowing the solutions to equilibrate at room temperature over 3 days. Thereafter, the saturated drug solutions were spiked with ^{3}H-β-estradiol to produce a specific activity of 0.1 μCi/mg. Solutions steroid concentrations were confirmed by liquid scintillation counting and stored at 0°C under nitrogen.

Abdominal female human skin from elective abdominoplasties was received within 4 hr of surgery, dermatomed to a thickness of 500 μm using a Pagetts electric dermatome, and stored for up to 2 weeks at 4°C in tissue culture medium (Dulbecca's modified Eagles medium with 5% fetal calf serum, Flow Laboratories, McLean, VA). Dermatomed skin was either grafted onto congenitally athymic rats (body weight, 200 g) as a skin sandwich flap within 24 hr of dermatoming or used over the following week for *in vitro* permeation experiments.

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In Vivo Percutaneous Absorption

Congenitally athymic (nude) rats (180–220 g in body weight) were purchased from the National Cancer Institute, Frederick, MD. The dermatomed human abdominal skin was grafted onto the nude rats in a three-step procedure described previously (13) to generate the human skin sandwich flap (HSSF).

Anesthesia and overall experimental conditions for *in vivo* percutaneous absorption experiments with the human skin sandwich flaps (HSSF) were performed as described previously (14). ^{3}H-β-Estradiol solutions were delivered to the human skin surface of the HSSF via a glass donor cell, the STeCC cell (14,15). Experiments were performed over a time period of 4–5 hr, with donor solution samples collected every hour and multiple samples collected periodically from both the local flap and the systemic blood circulations. All blood samples were treated as described previously (16) to quantitate the drug concentration in the various blood samples via liquid scintillation counting (Packard Model 1900CA, Downers Grove, IL). Three HSSF generated from independent skin sources were utilized for each vehicle tested.

Measurements of ^{3}H-β-estradiol flux *in vivo* were performed using both local flap and systemic drug concentrations, local blood flow as assessed by laser Doppler velocimetry [16], and surface area of the treated skin site:

$$\text{Flux } (\mu g\ cm^{-2}\ hr^{-1}) = \frac{\{[C_{flap} - C_{sys}](\mu g/mL) \times \text{blood flow } (mL/hr)\}}{\text{surface area } (cm^2)} \quad (1)$$

In vivo permeability coefficients (K_{papp}; cm hr^{-1}) were calculated from donor drug concentrations (C_d; μg mL^{-1}) in the SteCC cell and drug flux (μg cm^{-2} hr^{-1}) determined from Eq. (2).

$$J = C_d \times K_{papp} \quad (2)$$

Apparent diffusion coefficients (D_{mapp}; cm^2 hr^{-1}) were calculated from the independently measured K_{papp}, the apparent partition coefficient (K_{mapp}), and the thickness of the rate limiting barrier (h) by rearrangement of Eq. (3).

$$K_{papp} = (K_{mapp} \times D_{mapp})/h \quad (3)$$

In Vitro Percutaneous Absorption

Human stratum corneum was isolated by incubating dermatomed split-thickness human abdominal skin with trypsin type IX, pH 8.0, in a manner reported previously (14). Volume-based K_{mapp} values were measured with isolated human stratum corneum in the various estradiol solutions in triplicate as described by Parry *et al.* (18).

In vitro and *in vivo* percutaneous absorption experiments were performed with unmatched skin sources. Side-by-side diffusion cells having 3-mL-total volume receiving and donor chambers were utilized for the *in vitro* permeation experiments. Human skin was mounted in the side-by-side diffusion cell such that the stratum corneum faced the donor chamber. In the symmetrical experiments, the donor chamber was filled with the steroid in the oleic acid vehicle and the receiving chamber was filled with the oleic acid vehicle only. Asymmetrical *in vitro* experiments were performed with the drug in the oleic acid vehicle in one chamber and 75% EtOH in the chamber. All cells were magnetically stirred externally and maintained at 32°C with a circulating water bath. Samples from the donor chamber (5 μL) were collected every hour, while samples (200 μL) from the receiving chamber were collected periodically throughout 56 hr. Sample volumes removed from the receiving chamber were replaced with equal volumes of fresh 32°C vehicle. Radioactivity in the collected samples was determined by liquid scintillation counting.

Total amount of drug absorbed across skin *in vitro* is presented as the accumulated β-estradiol (μg/cm^2) normalized for solute concentration (μg/mL) in the various vehicles [(μg/cm^2)/(μg/cm^3) = cm].

In vitro K_{papp} and D_{mapp} were determined according to methods developed previously (18), in which the diffusivity and skin thicknesses were fit to the permeation data with a nonlinear fitting program using a non-steady-state mathematical model.

Skin Disposition of β-Estradiol

Disposition of β-estradiol in human skin layers *in vitro* and *in vivo* was determined by sectioning the 2- to 4-mm-diameter biopsy collected at the treated skin site using methods described previously (17). With this method, fresh biopsies are sectioned horizontal to the skin surface into ~100-μm skin wafers using cyanoacrylate, microscope cover slides, glass microscope slides, and a single-edged razor. Individual skin wafers were subsequently digested and analyzed for drug content by liquid scintillation counting.

Statistical Analysis

Statistical significance of the measured permeation parameters between vehicles in both *in vivo* and *in vitro* skin model systems was evaluated nonparametrically by Mann–Whitney U test using StatView II (Abacus Concepts, Calabasas, CA).

RESULTS

In vitro β-estradiol flux across human skin in symmetrical 75% EtOH (Table I) was similar to *in vivo* flux ($P > 0.05$). Symmetrical addition of 5% oleic acid to the 75% EtOH vehicle *in vitro* ($P < 0.001$) enhanced β-estradiol flux across human skin 6×. The addition of oleic acid to 75% EtOH vehicle *in vivo,* however, did not alter solute flux ($P > 0.05$).

The physicochemical parameters associated with *in vitro* and *in vivo* β-estradiol flux across human skin for these vehicles are elucidated in Table I. The addition of 5% oleic acid to 75% EtOH decreased β-estradiol solubility (C_d) 1.5× and increased the K_{mapp} 1.6×. These changes were associated with a 6× increase in D_{mapp} and a 10× increase in K_{mapp} *in vitro*. The *in vivo* $K_{p_{app}}$ of β-estradiol in the 5% oleic acid vehicle, however, was not different ($P > 0.05$) from that in the 75% EtOH vehicle.

The disparity in β-estradiol $K_{p_{app}}$ *in vitro* and *in vivo* in the 5% oleic acid vehicle reflects differences in the diffusion scenario between the two skin models. *In vitro* permeation

Table I. β-Estradiol Physicochemical Parameters in Human Skin *in Vivo* and *in Vitro*

Vehicle	C_d (mg/mL)[a]	K_{mapp}[b]	Flux (μg/cm² hr)[c]		K_{papp} ($\times 10^{-3}$ cm/hr)[d]		D_{mapp} ($\times 10^{-9}$ cm²/sec)[e]	
			In vivo	*In vitro*[f]	*In vivo*	*In vitro*[f]	*In vivo*	*In vitro*[f]
75% EtOH	10.0	0.34 ± 0.03 (36)	8.4 ± 1.8 (4)	6.5 ± 1.1 (5)	0.8 ± 0.2 (4)	1.0 ± 0.2 (5)	1.1 ± 0.2 (4)	3.2 ± 0.5 (5)
5% OA in 75% EtOH	6.1	0.54 ± 0.02 (54)	7.2 ± 2.4 (4)	40.7 ± 36.4*,** (5)	1.2 ± 0.4 (4)	10.0 ± 8.9** (5)	0.5 ± 0.2** (4)	20.6 ± 18.4 (5)

[a] Solubility.
[b] *In vitro* volume-based skin-to-vehicle apparent partition coefficient, mean ± SE (*n* skin sources in triplicate).
[c] Mean ± SE (*n*).
[d] Apparent permeability coefficient; mean ± SE (*n*).
[e] Apparent diffusion coefficient; mean ± SE (*n*).
[f] *In vitro* symmetrical side-by-side diffusion cells.
* $P < 0.001$ vs 75% EtOH.
** $P < 0.05$, *in vivo* vs *in vitro*.

experiments performed in symmetrical side-by-side diffusion cells have the same vehicle in both donor and receiving chambers, while the *in vivo* experiments are asymmetrical, with the donor chamber containing the solute and vehicle and the receiving chamber containing an aqueous vehicle, blood. The influence of symmetrical vs asymmetrical 5% oleic acid on normalized β-estradiol permeation (cm) across human skin *in vitro* is shown in Fig. 1A. Symmetrical 5% oleic acid *in vitro* increased β-estradiol permeation across human skin after ~12 hr, while the asymmetrical experiments demonstrated solute permeation similar ($P > 0.05$) to that measured in 75% EtOH only. Thus, β-estradiol permeation was increased *in vitro* only when oleic acid was equilibrated across the membrane.

The importance of oleic acid within the skin on β-estradiol permeation *in vitro* was further evaluated by preequilibrating split-thickness human skin in either 75% EtOH or 5% oleic acid for 24 hr at 32°C. Thereafter, the skin was removed from the respective vehicles, blotted dry, and mounted in a side-by-side diffusion cell with both donor and receiving chambers filled with 75% EtOH. Preequilibration of the skin with 5% oleic acid increased β-estradiol permeation *in vitro* 6× above the same skin source pretreated with 75% EtOH (Fig. 1B). In contrast, human skin pretreated with 75% EtOH produced profiles of β-estradiol permeation similar ($P > 0.05$) to those of untreated human skin in the same vehicle.

Normalized β-estradiol content in skin biopsies collected at the termination of *in vitro* permeation experiments was 4× greater in symmetrical 5% oleic acid (OA/OA) than in asymmetrical (OA/EtOH) experiments (Fig. 2). Normalized total β-estradiol skin contents in the asymmetrical 5% oleic acid (OA/EtOH) and symmetrical 75% EtOH (EtOH/EtOH) *in vitro* experiments, however, were similar ($P > 0.05$). The β-estradiol disposition in consecutive human skin layers in 75% EtOH and 5% oleic acid vehicles *in vivo* were also similar ($P > 0.05$) (Fig. 3). Thus, *in vitro* β-estradiol content in human skin was increased only when the skin was equilibrated with the fatty acid.

No significant difference ($P > 0.05$) in the D_{mapp} of β-estradiol in the 75% EtOH and 5% oleic acid vehicles was detected *in vivo* [1.1 ± 0.2 and 0.5 ± 0.2 × 10^{-9} cm²/sec (mean ± SE; $n = 4$), respectively] or in the asymmetrical *in vitro* experiments [3.2 ± 0.7 and 3.1 ± 0.2 × 10^{-9} cm²/sec ($n > 4$), respectively].

DISCUSSION

The similarity in β-estradiol flux across human skin from various concentrations of aqueous ethanol vehicles using symmetrical *in vitro* diffusion cells and the *in vivo* human skin sandwich flap model has previously demonstrated that *in vivo* solute flux could be predicted from *in vitro* experimentation (14). The 3000× increase in solubility and the 10× decrease in K_{mapp} of the solute in aqueous and 75% EtOH vehicles were critical to the elucidation of the physicochemical parameters responsible for the 30× ethanol-enhanced steroid flux (14).

Addition of 5% oleic acid to the 75% EtOH vehicle decreased β-estradiol solubility 1.5× and increased K_{mapp} 1.6× but did not change the stratum corneum thickness (h) as measured by engineer's micrometer (Mitutoyo 7326, Japan; sensitivity limits of 2.5 μm; data not shown). These physicochemical parameters were associated with a 6× increase in β-estradiol flux, a 10× increase in K_{papp}, and a 6× increase in D_{mapp} in the symmetrical *in vitro* experiments. In contrast, 5% oleic acid did not change the steroid K_{papp} or D_{mapp} or *in vivo* ($P > 0.05$) from that measured with 75% EtOH. Thus, while the mechanism of ethanol-increased β-estradiol flux across human skin *in vitro* reflected the solubility and the K_{mapp} into the stratum corneum, increased steroid flux in 5% oleic acid was more complex.

Disparity between the β-estradiol permeability coefficients *in vitro* and *in vivo* may reflect the different experimental time periods, unmatched skin sources, physical states of the skin sample over time, or nonuniform distribution of the vehicle within the skin *in vivo*. Although differences are observed in the current study between unmatched skin sources, the variabilities of the various parameters *in vitro* and *in vivo* are similar and are not different from those measured by others (4,19–23).

Compromise of the stratum corneum barrier integrity may also increase solute permeation across skin (24). The

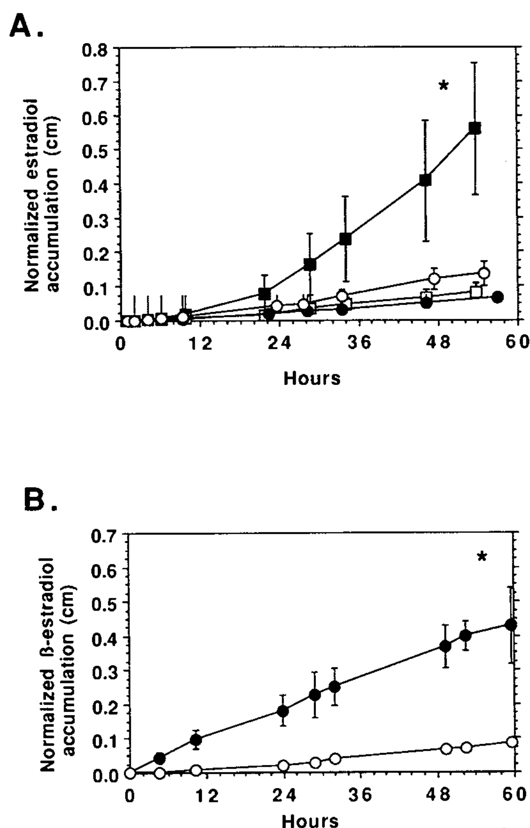

Fig. 1. (A) Influence of *in vitro* diffusion cell design on β-estradiol permeation across human skin from 5% oleic acid and 75% EtOH vehicles. Filled symbols represent symmetrical diffusion cell experiments; open symbols represent asymmetrical diffusion cell experiments. Filled squares—5% oleic acid in both donor and receiving chambers; open squares—5% oleic acid in donor chamber, 75% EtOH in receiving chamber; filled circles—75% EtOH in both donor and receiving chambers; open circles—5% oleic acid in the receiving chamber, 75% EtOH in the donor chamber. Mean ± SE for $n = 4$ skin sources. (*) $P < 0.05$ vs symmetrical 75% EtOH. (B) Effect of 5% oleic acid or 75% EtOH skin pretreatment on β-estradiol permeation across human skin *in vitro*. Open circles—with 75% EtOH; filled circles—with 5% oleic acid in 75% EtOH pretreatment. Mean ± SE for $n = 3$ skin sources. (*) $P < 0.05$ from 75% EtOH pretreatment.

presence of lag times and a linear normalized accumulation of β-estradiol across human skin from 24 to 60 hr *in vitro* with both vehicles however, demonstrate an intact barrier. *In vivo* β-estradiol flux from both vehicles was also constant from 1 to 4 hr (data not shown). Further, all physicochemical parameters were measured under steady-state conditions in both model systems, despite the longer time lag differences required *in vitro*.

The disparity in β-estradiol K_{papp} in 5% oleic acid *in vitro* and *in vivo* likely reflects differences in oleic acid distribution in the human skin. While symmetrical *in vitro* diffusion cells are commonly used to evaluate transdermal solute permeation due to the minimal changes in vehicle composition, vehicle gradients, and osmotic forces across the skin, they do not represent the asymmetrical scenario *in vivo*, in which the solute in an organic delivery vehicle is applied to the stratum corneum and the receiving chamber,

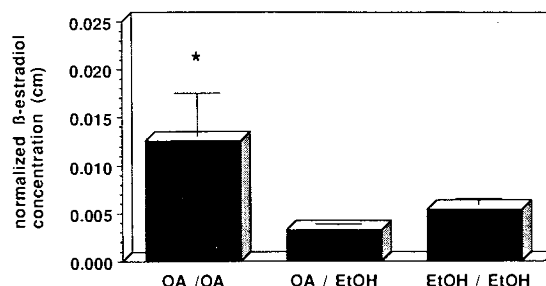

Fig. 2. Comparison of β-estradiol content in human skin *in vitro* following permeation in 75% EtOH and 5% oleic acid vehicles in symmetrical and asymmetrical diffusion cells. OA/OA—5% oleic acid in both donor and receiving chambers; OA/EtOH—5% oleic acid in the donor chamber, 75% EtOH in the receiving chamber; EtOH/EtOH—75% EtOH in both donor and receiving chambers. Mean ± SE for $n = 3$ skin sources. (*) $P < 0.05$ from EtOH/EtOH.

the dermis, is in intimate contact with aqueous fluid, the blood. *In vivo* conditions produce a concentration gradient of both the vehicle and the solute across the skin. These concentration gradients across skin can be established *in vitro* using an asymmetrical permeation design. The K_{papp} of β-estradiol across human skin in asymmetrical 5% oleic acid experiments was indistinguishable ($P > 0.05$) from that measured *in vivo*. Further, the K_{papp} of β-estradiol in these asymmetrical *in vitro* and *in vivo* experiments with 5% oleic acid was similar to that with 75% EtOH ($P > 0.05$). Thus, while a 6× increase in β-estradiol permeation occurred in human skin equilibrated with 5% oleic acid, it did not occur *in vivo* or *in vitro* in the presence of an oleic acid concentration gradient across the skin. These data agree with previously published work, in which solute-enhanced permeation with oleic acid was concentration dependent (7,11,25).

It is interesting that the oleic acid concentration-dependent enhancement of β-estradiol permeation *in vitro* was independent of the isolated stratum corneum-to-vehicle K_{mapp} or β-estradiol solubility. These data suggest that absorption of this hydrophobic solute may be stratum corneum independent and more controlled by diffusion through the epidermal/dermal skin layers. This concept agrees with previous studies of nonpolar solutes (8,25), where the total skin diffusion was the rate-limiting barrier (26). Enhanced permeation of a hydrophobic solute would therefore require greater oleic acid concentrations and solute in the deeper skin layers. Indeed, the β-estradiol content in human skin

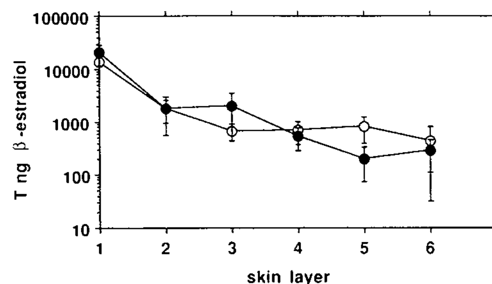

Fig. 3. *In vivo* β-estradiol disposition through grafted human skin in 75% EtOH and 5% oleic acid vehicles. Open circles—75% EtOH; filled circles—5% oleic acid. Mean ± SE; $n = 4$. Each skin layer is ~100 μm thick.

biopsies collected from the symmetrical 5% oleic acid *in vitro* experiments was 4× greater than in asymmetrical 5% oleic acid or symmetrical 75% EtOH experiments and was associated with a 3× increase in the K_{papp} of β-estradiol above that measured in asymmetrical *in vitro* permeation experiments ($10.2 \pm 4 \times 10^{-3}$ vs $3.3 \pm 0.1 \times 10^{-3}$ cm/hr, respectively). These collective data suggest that β-estradiol copermeates with oleic acid. This hypothesis is supported by the similar K_{papp} of oleic acid alone (data not shown) and β-estradiol in the 5% oleic acid vehicle in the asymmetrical *in vitro* diffusion experiments ($3.3 \pm 0.1 \times 10^{-3}$ vs $4.7 \pm 1.7 \times 10^{-3}$ cm/hr, respectively).

These data are in contrast to those mechanisms suggested previously (7,11,12) in which oleic acid-enhanced permeation of solutes in ethanol result from the disruption of intercellular lipid bilayers, increase in lipid fluidization, and solute diffusivity within the stratum corneum. *In vivo* and asymmetrical *in vitro* D_{mapp} values of β-estradiol in 5% oleic acid and 75% EtOH in the present studies, however, are similar ($P > 0.05$). Thus, 5% oleic acid does not alter β-estradiol permeation *in vivo* or asymmetrically *in vitro* by altering skin diffusivity.

The lack of oleic acid enhanced β-estradiol permeation in the current asymmetrical *in vitro* and *in vivo* experiments was nonetheless surprising, with regard to other studies (7,26) using asymmetrical modified Franz cells, in which permeation enhancement of a variety of solutes was oleic acid concentration dependent. Oleic acid-enhanced permeation however, was associated with a finite dose in propylene glycol using cadaver skin. The present study evaluated oleic acid permeation enhancement of a hydrophobic solute, β-estradiol, with an infinite dose, in an ethanol vehicle, using fresh human abdominal skin. Finite dosing of a binary enhancer vehicle plus solute may have profound effects on their respective skin disposition, with the more rapid absorption of propylene glycol creating increased oleic acid concentrations on the skin surface. Increasing oleic acid concentration on the skin surface will produce a greater driving force of oleic acid across skin and, thus, a higher disposition within skin. An infinite dose maintains a constant-concentration driving force of oleic acid and solute across skin, thus obviating alterations in oleic acid thermodynamic activity on the skin surface. Oleic acid or β-estradiol uptake into and permeation across cadaver skin from various anatomical locations (27) versus fresh abdominal skin may also differ. Further, oleic acid has been demonstrated to enhance hydrophilic solute permeation more than hydrophobic solutes (7,26). These data, in conjunction with the present work, support a limited role of transdermal oleic acid as a permeation enhancer for hydrophobic solutes.

The present investigation demonstrates that oleic acid is not a universal permeation enhancer. Permeation enhancement with this fatty acid will be influenced by solute hydrophilicity, the base vehicle in which oleic acid and solute are solubilized, finite vs infinite dosing, and the skin model systems used to evaluate permeation. Enhanced β-estradiol permeation with oleic acid in the current study was tissue concentration dependent and increased *in vitro* only when skin was equilibrated with oleic acid. Generation of an oleic acid concentration gradient through the skin did not change β-estradiol permeation *in vitro* or *in vivo* from that in the base 75% EtOH vehicle. Prediction of *in vivo* β-estradiol permeation across human skin in the 5% oleic acid vehicle was possible, however, using the asymmetrical *in vitro* data. These data highlight the necessity of understanding permeation mechanisms *in vivo* when potential permeation-enhancing vehicles are evaluated *in vitro*. Design of the *in vitro* diffusion cell experiment with regard to its relevancy to the *in vivo* diffusion scenario and dosing format (topical versus transdermal) is imperative for using *in vitro* results to predict *in vivo* use.

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