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Report

Mechanism of Ethanol-Enhanced Estradiol Permeation Across Human Skin *in Vivo*

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The influence of ethanol on the permeation of 17β-estradiol (estradiol) across viable human skin *in vivo* was investigated with the human skin sandwich flap model. Maintaining continuous delivery of a constant concentration of the solute in phosphate-buffered saline, pH 7.4 (PBS), or mixtures of ethanol in PBS to the skin surface revealed that steady-state flux of estradiol was achieved within 30–60 min and maintained throughout 4 hr. The 10-fold decrease in *in vivo* flux and permeability coefficient (K_p) of tracer estradiol solutions in ethanol or ethanol solutions compared with PBS vehicle reflected the 10-fold difference in the apparent partition coefficients (K_m) of estradiol from the respective vehicles into isolated human stratum corneum. Neither the stratum corneum thickness nor the diffusion coefficient of estradiol was significantly different among the vehicles tested. *In vivo* flux of estradiol in ethanol or ethanol solutions across viable human skin was increased with saturated solutions of estradiol. Further, *in vivo* flux of estradiol from vehicles such as PBS, ethanol, and ethanol mixtures, which minimally alter the rate-limiting barrier, can be successfully predicted with knowledge of only two physicochemical parameters, the estradiol concentration in the vehicle and the K_m of estradiol from the vehicle into isolated human stratum corneum.

KEY WORDS: human; skin; estradiol; ethanol; percutaneous absorption; permeation enhancers.

INTRODUCTION

The stratum corneum barrier of skin limits the transdermal permeation of some drugs, resulting in insufficient systemic concentrations for therapeutic efficacy. Enhancing the permeability of a compound across the stratum corneum is being pursued with the hope of achieving greater systemic concentrations of the therapeutic solute. Transdermal delivery of a particular solute may be determined in part by its physicochemical characteristics; lipophilicity, molecular weight, partition coefficient from the vehicle of choice into skin, and solubility of the compound within the chosen vehicle (1). Increasing the permeability of a solute across the skin may in theory be accomplished by changing the vehicle to increase solubility of the solute or altering the skin resistance to permeation (2,3).

A variety of vehicles have been identified as "permeation enhancers" (4–16). Ethanol in particular has been observed to increase the permeation of a number of compounds (2,4,14,16). The focus of the present investigation was to study the mechanisms of the ethanol enhanced *in vivo* permeation of 17β-estradiol (estradiol) across human skin. This was accomplished using the human skin sandwich flap model (HSSF) (17). This model system allows quantification of a vehicle's influence on estradiol flux across viable human skin, in addition to the evaluation of the systemic blood concentration. Alterations in the permeability and apparent diffusion coefficients of estradiol from a variety of vehicles may be quantitatively assessed with the model. These coefficients can be compared with additional physicochemical parameters, such as the apparent partition coefficient (K_m) of estradiol from the vehicle into isolated human stratum corneum and the solubility of estradiol in the vehicles.

Understanding the possible *in vivo* mechanisms of ethanol on estradiol permeation, which may include "co-permeation" of the solute with the vehicle, requires the knowledge of solute activity in the vehicle over the experimental time frame. The continuous *in vivo* delivery of estradiol in the various vehicles to the skin surface is accomplished with a STeCC (stirred, temperature-controlled constant concentration) donor cell developed in this laboratory (18). Thus, the STeCC donor cell in conjunction with the human skin sandwich flap enables the differentiation of the vehicle effects on *in vivo* permeation.

MATERIALS AND METHODS

Animals. Congenitally athymic (nude) rates (180–220 g) were purchased from the National Cancer Institute, Frederick, MD. HSSF with an isolated but accessible vasculature were generated as an island skin sandwich. The flap was generated by first grafting a split thickness (0.5-mm) human skin graft obtained from elective abdominoplasties to the subcutaneous surface of a skin flap on the abdomen of a

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nude rat. The skin sandwich flap was then isolated on its arterial and venous vasculature and transferred to the back of the animal via a subcutaneous tunnel and sutured in place. A total period of 5 weeks was required to generate a skin sandwich flap that was ready for experimentation. Two weeks were allowed between experiments on the same skin sandwich flap to allow recovery from the surgical procedures utilized in the experiment.

Animals were housed in individual sterilized polycarbonate cages with sterilized bedding in a clean double-filtered animal facility isolator (Duo-Flow, Lab Products) which utilizes a 12-hr light cycle. Food and water (pH 2.5) were sterilized and allowed ad libitum. All animals were removed from subcutaneous cyclosporine A (Sandimmune; i.v. solution, Sandoz, East Hanover, NJ) 2 days prior to experimentation.

Chemicals. [6,7-^{3}H(N)]17β-Estradiol (sp act, 60 Ci/mmol) in ethanol was greater than 98% purity as purchased from New England Nuclear Research Products, Dupont, Boston, MA. Crystalline estradiol was purchased from Sigma, St. Louis, MO. Ethanol (Gold Label; 95%) was used as purchased from Aldrich (Milwaukee, WI). Tracer (0.30 μg/ml) and saturated solutions of estradiol in 95% ethanol, PBS, pH 7.4 (PBS), or 75% (v/v) ethanol:PBS were prepared with unlabeled estradiol and ^{3}H-estradiol (sp act, 120 μCi/mmol) to investigate the relative influence of vehicle and solute concentration on the permeation process.

STeCC Donor Cell. The donor cell utilized in the present experiments was designed to minimize the diffusional boundary layer, maintain the temperature of the solute solution, and provide a constant concentration of solute at the exposed skin surface (18). The diffusional surface area of the internal cell was 0.8 cm^2. The internal cell volume was 1 ml, and the total circulating volume of the solute solution was 6 ml.

Experimental Protocol for Percutaneous Absorption Experiments. The nude rats with HSSF were initially anesthetized intraperitoneally with 100 mg/kg Ketalar (Parke-Davis, Morris Plains, NJ) and maintained throughout a 4-hr experimental time period with additional doses of 30 mg/kg every 45 min. The various ^{3}H-estradiol solutions were dispensed from a 10-ml disposable syringe positioned in a Harvard microliter syringe pump (Harvard Apparatus Bioscience, South Natick, MA). The STeCC donor cell was mechanically fixed on the HSSF and attached to the skin surface with a medical-grade silicone adhesive (gift from the Center of Engineering Design, University of Utah, Salt Lake City, UT). The radiolabeled estradiol solutions were pumped through the STeCC donor cell at a flow rate of 1.0 ml/hr over a period of 4 hr and collected in a capped receiving reservoir. Samples of the donor solution were sampled every hour from the STeCC donor cell efferent port.

A total of seven local flap and five systemic whole blood samples (40 μl each) were collected into heparinized microhematocrit tubes every 30 min over a 4-hr experiment. Initial studies demonstrated that systemic blood concentrations of estradiol did not change significantly from background over the 4-hr experimental time period. Therefore, sampling the systemic circulation each hour was adequate to monitor this compartment. Additional blood samples from the venous flap were collected to ascertain the lag time of estradiol flux *in vivo*. All blood samples were treated as previously described (19) and the radioactivity within the various blood samples was quantitated with a liquid scintillation counter (LSC Beckman Model LS 8100; Beckman, Irvine, CA).

The three HSSF utilized for each vehicle tested in the study were generated from independent sources of human skin. Each HSSF served as its own control. Permeation experiments on each HSSF were performed every 2 weeks.

In Vivo *Calculations.* The local circulating blood in the HSSF *in vivo* model represents the initial sink for solute absorption across the skin, with the ultimate sink being the systemic circulation. Blood in the skin sandwich flap eventually circulates into the systemic venous circulation. Therefore, accumulation of solute in the local flap venous blood is not observed over time. However, the systemic blood eventually recirculates through skin sandwich flap as arterial blood containing previously absorbed solute. Quantitation of total solute absorption across the skin sandwich flap thus requires knowledge of the flap (immediately draining the skin sandwich flap) and systemic blood concentrations of the solute (C_{flap} and $C_{systemic}$, respectively), the instantaneous blood flow to the treated skin site, as well as the surface area of skin exposed to the solute to calculate instantaneous flux [Eq. (1)]:

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

Blood flow to the treated skin site on the HSSF was monitored throughout the experiment with a laser Doppler velocimeter (MedPAcific, Seattle, WA) with specific notation of the blood flow at all blood collection time points. The millivolt analog readout was converted into a volume per time unit (ml/hr) via a correlation with a blood flow meter (Micron Medical, Los Angeles, CA) as previously described (19). Concentration of the drug in the blood samples collected was calculated using the disintegrations per minute (dpm) of the individual samples counted in the LSC, the molecular weight (MW) of the compound (g/mol), the specific activity (SA) of the drug solution (μCi/μmol), the volume (VOL) of blood (ml), and a conversion factor (2.22 × 10^6 dpm/μCi) of dpm into microcuries (μCi).

$$\mu g/ml = \frac{(dpm)(MW)}{(2.22 \times 10^6)(SA)(VOL)}$$

Apparent Partition Coefficients Experiments. Human stratum corneum was isolated from split thickness human abdominal skin using a 1% trypsin (type IX; Sigma, St. Louis, MO), pH 8.0, solution at 40°C for 2 hr (20). Enzyme activity was terminated with 2 ml of 5% albumin. Thereafter, the isolated stratum corneum sheets were rinsed five times in distilled water. Six-millimeter-diameter biopsies were generated from the sheets of isolated stratum corneum with a 6-mm disposable punch biopsy (Accuderm, Ft. Lauderdale, FL) and stored desiccated under vacuum on 25 × 10-mm disposable petri dishes. Light microscopy of the isolated stratum sheets confirmed the absence of epidermis in the stratum corneum skin sample.

Three samples from each skin source (n = 18) were incubated with 2 ml of saturated solutions (25°C) of estradiol

spiked with ^{3}H-estradiol in the vehicles of choice for 24 hr at 25°C. The stratum corneum samples (each ~0.2 mg desiccated dry weight) were then removed from the solutions, rinsed three times in distilled water, and blotted dry on Whatman No. 1 filter paper. Rinsing the 6-mm-diameter stratum corneum biopsies in 10 ml of distilled water or methanol did not significantly alter the K_m. The stratum corneum samples were digested with tissue solubilizer similar to the blood samples and submitted to LSC.

Apparent partition coefficients (K_m) of estradiol from the various vehicles into isolated 6-mm-diameter human stratum corneum disks were calculated from the estradiol concentrations in the stratum corneum and solutions based upon volume (K_m). The concentration of solute within the stratum corneum was determined by dividing the amount of radiolabeled drug in the stratum corneum by the stratum corneum volume (cm^3 or ml). The volume was calculated from the thickness of the stratum corneum measured after incubation in the vehicle (cm) and surface area (cm^2). The coefficient of variation of apparent K_m within the same skin source was less than 7%.

Stratum Corneum Thickness Determinations. Human abdominal stratum corneum thicknesses (h) were measured with an Engineer's micrometer with a sensitivity limit of 2.5 μm (Mitutoyo No. 7326, Japan). The isolated human stratum corneum thicknesses were analyzed after 2, 6, 8, and 24 hr of incubation with the various vehicles. The average hydrated (in the presence of vehicle) stratum corneum thickness (42 ± 8.4 μm; $X \pm$ SD, $n = 12$) was not significantly altered by the different vehicles tested.

Permeability Coefficients. Permeability coefficients (K_p) were calculated from the *in vivo* data using the steady-state flux, J, and the donor concentration (C_d) of the estradiol solutions [Eq. (2)]:

$$K_p = J/C_d \quad (2)$$

Apparent Diffusion Coefficients. Apparent diffusion coefficients (D) were calculated using the K_m and h, as determined *in vitro* with human abdominal stratum corneum, and K_p determined *in vivo* across HSSF [Eq. (3)]:

$$D = (K_p)(h/K_m) \quad (3)$$

RESULTS

STeCC Cell

Maintaining a constant estradiol concentration in ethanol over the experimental time period is requisite to understanding the influence of the ethanol vehicle on estradiol flux across human skin *in vivo*. It is necessary, therefore, to develop a donor cell for *in vivo* experiments, which would be light in weight to prevent alteration in the cutaneous circulation, well stirred to prevent diffusional boundary layers, and temperature controlled and would continuously deliver solute to the skin surface at the same activity. The infusion pump via the STeCC cell delivers a constant concentration of radiolabeled estradiol in tracer and saturated solutions (Fig. 1) which results in constant flux of the permeating solute across the grafted human skin of the HSSF over the 4-hr experimental time period as represented in Fig. 2.

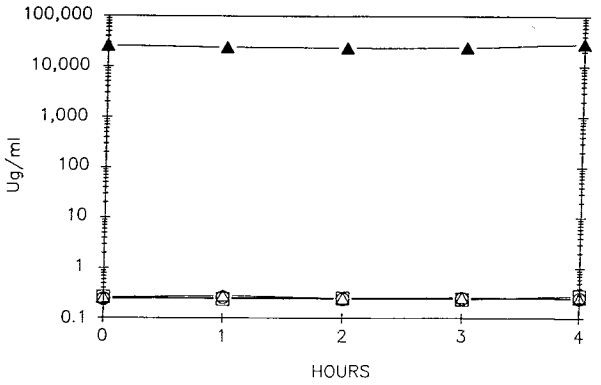

Fig. 1. Constant STeCC cell delivery of estradiol concentration (μg/ml) to the skin surface of a human skin sandwich flap from a tracer PBS solution (open circles), a tracer 95% ethanol solution (open triangles), and saturated 95% ethanol solution (filled triangles) over a 4-hr experimental time period.

Physicochemical Parameters

The flux of tracer concentrations (0.31 μg/ml) of radiolabeled estradiol in PBS across human skin in the HSSF is 10-fold greater than the flux in 95% ethanol (Table I) and 30-fold greater than the flux in 75% (v/v) ethanol:PBS. Increasing the concentration of estradiol to saturation in 95% ethanol (31.1 mg/ml) results in a 3000-fold increase in flux above that observed with the tracer estradiol solutions.

The K_p of estradiol across human skin *in vivo* in tracer ethanol solutions is 10-fold less ($P < 0.05$) than that observed with PBS (Table I), reflecting the differences in apparent skin-to-vehicle partitioning (K_m) of estradiol in human stratum corneum. The K_p of estradiol across human skin from saturated solutions in ethanol does not differ significantly from the tracer solutions. Further, the K_p of estradiol in 75% (v/v) ethanol:PBS is similar to that observed with estradiol in 95% ethanol. The apparent diffusion coefficients (D) calculated using apparent K_m, a skin thickness, h, of 40 μm, and K_p from the *in vivo* permeation experiments suggests that skin diffusivity is not significantly altered in the presence of ethanol ($P > 0.05$). Partition coefficient experiments reveal

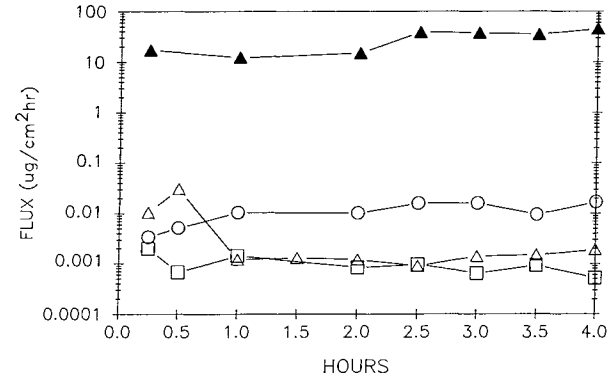

Fig. 2. *In vivo* flux of estradiol from a variety of vehicles across a representative human skin sandwich flap from the STeCC donor cell containing a tracer estradiol solution in PBS, pH 7.4 (open circles), tracer estradiol solution in 95% ethanol (open triangles), tracer estradiol solution in 75% ethanol (open squares), and saturated estradiol solution in 95% ethanol (filled triangles) over a 4-hr experimental time period. Mean and standard error of the mean for $n = 3$.

Table I. Influence of Ethanol Concentration on Estradiol Physicochemical Parameters in Human Skin

Vehicle	C_d (mg/ml)	Flux (ng/cm^2 hr)[a]	K_p (cm/hr)[a]	D (cm^2/sec)[a]	K_m[b]
PBS, pH 7.4	3.1×10^{-4}	17.0	6.5×10^{-2}	5.1×10^{-10}	9.0
		(8.6)	(1.7×10^{-2})	(1.4×10^{-10})	(0.8)
95% Ethanol	3.1×10^{-4}	1.7*	5.1×10^{-3}*	5.1×10^{-10}	0.7*
		(1.0)	(2.9×10^{-3})	(2.9×10^{-10})	(0.1)
75% Ethanol	3.1×10^{-4}	0.6*	2.0×10^{-3}*	1.6×10^{-10}	0.8*
		(0.4)	(1.2×10^{-3})	(1.0×10^{-10})	(0.2)
95% Ethanol	31.1	53,900*	1.9×10^{-3}*	1.9×10^{-10}	0.7*
		(4,500)	(0.6×10^{-3})	(5.5×10^{-11})	(0.1)
25% Ethanol	0.6				10.6
					(1.5)
50% Ethanol	1.5				2.6*
					(0.9)
75% Ethanol	10.0				0.8*
					(0.2)

[a] $X \pm$ SE; $n = 3$.
[b] K_m based on volume; $X \pm$ SE; $n \geq 5$.
* $P < 0.02$ from PBS pH 7.4 vehicle by Welch's t test.

that skin-to-vehicle partitioning (K_m) of estradiol in PBS is 10-fold greater than in the ethanol solutions. In contrast, the solubilities of estradiol increase with increasing ethanol concentration, e.g., estradiol solubility in PBS, 95% ethanol, and 75% (v/v) ethanol:PBS was 0.003, 10, and 31.1 mg/ml, as given by Ghanem *et al.* (14).

Correlation of the Apparent K_m with Estradiol Solubility

The skin-to-vehicle apparent K_m of estradiol in stratum corneum in tracer solutions dominates the K_p of the drug across human skin *in vivo*, since apparent D and h are not significantly altered in the presence of ethanol. The percentage of ethanol in the vehicle has been demonstrated to influence greatly the solubility of estradiol (14); the greater the ethanol content in the vehicle, the greater the solubility. Further, the solubility of estradiol in the vehicles tested inversely correlates with the skin-to-vehicle apparent K_m of estradiol, albeit not proportionately (Fig. 3).

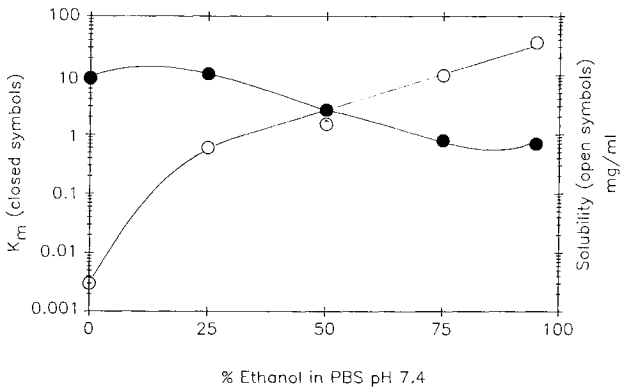

Fig. 3. Influence of ethanol content in the PBS vehicle on estradiol solubility (open symbols) and the partition coefficient (K_m) (filled symbols) into isolated human stratum corneum sources. Mean and standard error of the mean for $n \geq 5$.

Correlation of the Apparent K_m and Donor Concentration with Estradiol Flux

The flux of radiolabeled estradiol across the HSSF from tracer ethanol solutions is 10-fold less than that observed with tracer solutions of PBS. The decrease in flux of estradiol from ethanol solutions directly reflects the decrease in apparent K_m of the solute into human stratum corneum. However, saturated ethanol solutions of estradiol produce a 30,000-fold increase in estradiol flux above tracer concentrations in ethanol. Therefore, the solubility of estradiol in the vehicle and the apparent K_m are critical to the resulting flux across human skin *in vivo*.

A log/log plot (Fig. 4) of the product of apparent partitioning (K_m and donor concentration (C_d) of estradiol in the various tested vehicles ($K_m \times C_d$) and *in vivo* flux describes their interrelationship ($r = 0.9954$). If flux is a function of the concentration of solute in the skin (C_m), the apparent D, and the thickness of the rate-limiting barrier (h) [Eq. (4)]:

$$\text{flux} = D \times C_m/h = D(K_m \times C_d)/h \quad (4)$$

then the log of this equation is given as [Eq. (5)]

$$\log \text{flux} = \log D - \log h + \log (K_m \times C_d) \quad (5)$$

If the thickness of the stratum corneum (h) and apparent D does not change significantly in the presence of the vehicles being studied, then the log function of flux is equal to the log product of K_m and C_d. The slope of the correlation in Fig. 4 is 1, suggesting that the apparent diffusivity of the stratum corneum is not appreciably altered by the presence of ethanol.

DISCUSSION

The influence of ethanol on the permeation of estradiol across grafted human skin was investigated using the HSSF, which allows quantitation of estradiol flux, K_p, and apparent D in viable human skin *in vivo*. Maintaining continuous delivery of a constant tracer estradiol concentration to the hu-

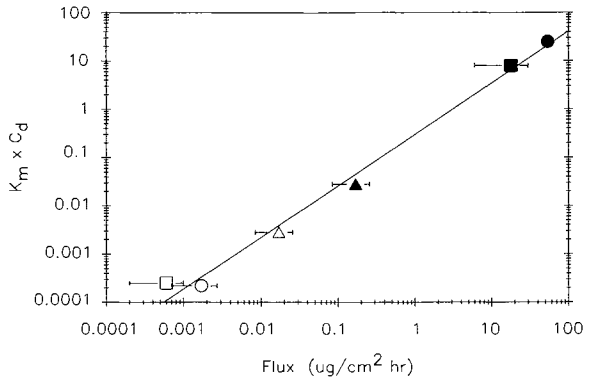

Fig. 4. Correlation of *in vivo* flux of estradiol across the human skin sandwich flap and the product of estradiol concentration (C_d) and partition coefficient (K_m) with a tracer estradiol solution in PBS, pH 7.4 (open circle), a tracer estradiol solution in 95% ethanol (open triangle), a tracer estradiol solution in 75% ethanol (open squares), and the respective saturated solutions (filled symbols). Mean and standard error of the mean ($n \geq 3$) for all vehicles tested.

man skin surface with the STeCC donor cell demonstrates that a steady-state flux of estradiol is achieved within 30–60 min and is maintained throughout a 4-hr period. Using the same concentration of estradiol in different vehicle solutions enables the influence of a particular vehicle solution on K_p to be determined. An alteration in the stratum corneum by the vehicle should therefore be reflected in an alteration in the K_p of the solute. Tracer concentration of estradiol in PBS and 95% ethanol, as well as 75% (v/v) ethanol:PBS, demonstrates that ethanol or ethanol solutions significantly ($P < 0.05$) decreases flux and K_p, due primarily to the decrease in apparent partitioning into human stratum corneum. The influence of contributing variables to the altered K_p may be further defined by evaluating the individual physicochemical parameters, apparent K_m, apparent D, and h [Eq. (6)]:

$$K_p = K_m \times D/h \quad (6)$$

In vitro analysis of the apparent partitioning coefficient of estradiol from ethanol into isolated human abdominal stratum corneum is 10-fold less than those in PBS. Apparent D and h are not significantly altered ($P > 0.05$) in ethanol solutions. Therefore, ethanol decreases the permeability of tracer solutions of estradiol across human skin *in vivo* 10-fold as a result of the decrease in apparent partitioning of the solute from ethanol into human stratum corneum.

In vitro studies utilizing hairless mouse skin in side-by-side diffusion cells by Ghanem *et al.* (10) demonstrate that the K_p of tracer solutions of ^{3}H-estradiol in PBS is 1.8×10^{-2} cm/hr, are similar to that observed in the present *in vivo* human studies (6.5×10^{-2} cm/hr). Increasing the ethanol concentration of the vehicle to 75 or 95% (v/v) ethanol:PBS decreases K_p in the hairless mouse skin studies to 3.6×10^{-4} and 9.0×10^{-4} cm/hr, respectively. The apparent K_m of estradiol from ethanol solutions into isolated hairless mouse stratum corneum shows similar trends (13). In contrast, ethanol solutions decrease the K_p only 10-fold in human skin *in vivo*, reflecting the 10-fold decrease in the apparent K_m measured in human stratum corneum.

Saturated solutions of estradiol differentiates the influence of solubility on permeation (2). Estradiol is 12,000-fold more soluble in 95% ethanol than PBS, which is reflected in the associated increased flux *in vivo*. The apparent D, h, and K_p of estradiol are not affected by the solute concentration. The increased flux observed with saturated solutions of radiolabeled estradiol in 95% ethanol, therefore, reflects the C_d on the passive diffusion of the solute across the skin. The influence of solute solubility on flux has also been noted *in vitro* with theophylline across hairless mouse skin (3).

Enhanced permeation of solutes across skin *in vitro* in the presence of ethanol has been hypothesized to be possibly affected by the extraction of skin lipids by ethanol (21). Regardless of a possible extraction of skin lipids with long-term ethanol exposure, the apparent diffusivity is not significantly altered in these *in vivo* studies. The K_p of tracer concentrations of estradiol in ethanol demonstrate that the vehicle actually decreases K_p due to a corresponding decrease in apparent K_m. Flux of estradiol across viable human skin is increased *in vivo* only with saturated solutions of estradiol, reflecting the increased solubility of estradiol in ethanol and, therefore, the increased concentration gradient across the skin. These observations are consistent with those by Good *et al.* (2). The present investigation further demonstrates that flux of estradiol across human skin *in vivo* can be successfully predicted from either PBS or ethanol vehicles with the knowledge of only two physicochemical parameters, the apparent partitioning of the solute into human stratum corneum and the solute concentration in the vehicle.

NOMENCLATURE

HSSF	Human skin sandwich flap
K_p	Permeability coefficient (cm/hr)
K_m	Partition coefficient, based on volume
D	Diffusion coefficient (cm^2/sec)
J	Flux (ng/cm^{2}hr)
h	Stratum corneum (μm)
C_d	Solute concentration in the donor vehicle (mg/ml)
C_m	Solute concentration in the skin (μg/ml)
C_{flap}	Concentration of the solute in the skin sandwich flap blood (μg/ml)
C_{sys}	Concentration of the solute in the systemic blood (μg/ml)
MW	Molecular weight (g/mol)
SA	Specific activity (μCi/μmol)
VOL	Volume of blood (ml)

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