Skin Penetration Flux and Lag-Time of Steroids Across Hydrated and Dehydrated Human Skin *in Vitro*

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To study the effect of hydration on skin absorption, we investigated penetration across human skin of twelve model chemicals having steroidal structure but different molecular weight and compared the steady-state penetration rate (J) and lag-time (t) across hydration intact skin (J_h and t_h) with that across dehydrated intact skin (J_d and t_d). Stratum corneum (SC) thickness of hydrated (52 μm) is 3.3 times that of dehydrated skin (16 μm). Transepidermal water loss (TEWL) of hydrated (7.6±2.1 g/m^2/h) is twice that of dehydrated skin (3.4±1.6 g/m^2/h, $p<0.05$) which are similar to *in vivo* values, suggesting the SC barrier function was recovered. The ratio of J_h/J_d ranged between 0.7 and 3.6 (average of 1.9). On the other hand, the ratio of t_h/t_d was almost constant (average of 0.8). Ratios of J_h/J_d and t_h/t_d were independent of MW and $K_{o/w}$. In percutaneous absorption experiments *in vitro*, skin was preserved in culture medium until use and SC might swell during that time. Therefore, we consider the possibility that J and t varied between hydrated and dehydrated skin. We confirmed the difference of J and t between hydrated and dehydrated skin *in vitro* and now need to define these results under *in vivo* condition.

Key words hydrated skin; dehydrated skin; penetration flux; lag-time; *in vitro* experiment; transepidermal water loss (TEWL)

Skin has a outermost thin layer, stratum corneum (SC), and underlayed viable epidermis and dermis. Because the SC is highly lipophilic, dry and a relatively effective percutaneous barrier, skin penetration is influenced by physicochemical properties of compounds. Hydrophobic compounds penetrate more easier than hydrophilics and lower than high molecular weight.[1] Relationships between penetration flux and physicochemical properties include: lipophilicity,[2] melting point,[3] molecular weight,[1,4] and pH of skin and vehicle.[5]

Water content of SC is 30 to 50% (w/w) of SC dry weight *in vivo*[6] and is varied when occluded by a water impermeable membrane and stored into water (*e.g.* phosphate buffered saline) to be used to skin transplantation and percutaneous absorption experiments *in vitro*. Increasing SC hydration alters barrier function, hence often increasing percutaneous absorption *in vitro*.[7] Bucks and Maibach, reporting the effect of occlusion on percutaneous absorption in human *in vivo*, discussed that the skin occlusion caused SC hydration did not necessarily increase percutaneous absorption of hydrophilic compounds.[8] Thus, the SC hydration is a important factor to reveal the *in vivo*/*in vitro* correlation of percutaneous absorption.

This study investigated a procedure to prepare dehydrated skin from hydrated skin stored in culture medium for some days and the effect of skin hydration on skin penetration of twelve steroids as model chemicals because they had a similar basic structure and different molecular weight.

MATERIALS AND METHODS

Materials We selected twelve steroids having a different molecular weight. Physicochemical properties of these chemicals were summarized in Table 1. Polyethylene glycol 400 (PEG400) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Methanol (HPLC grade), acetonitrile (HPLC grade), and distilled water (HPLC grade) were purchased from Fisher Chemicals (Fair Lawn, NJ, U.S.A.).

Skin Preparation Human cadaver skin samples (leg or back) were obtained from 39 Caucasian males between the age of 21 and 81 years old (average age with S.D., 53.9±15.6 years) at the Northern California Transplant Bank (Oakland, CA, U.S.A.). The skin samples were kept in MEM Eagle's medium with Earle's BSS (MEM medium) at 4 °C. SC of skin samples was hydrated because of preservation in MEM medium more than 2 d and we set the limitation of skin use on 5 d to avoid skin deterioration. We prepared three types of samples: stripped skin, hydrated intact skin and dehydrated intact skin. Stripped skin was obtained by SC stripping thirty times using adhesive tape (#1527-1, 3M Health Care). Hydrated intact skin was immediately used of storage in MEM medium. Dehydrated intact skin was prepared as follows: hydrated intact skin was placed on a 20 ml sample

Table 1. Physicochemical Properties of Model Chemicals

	M.W.	$\log K_{o/w}$	Cs in 40% PEG400 [μg/ml][b]
ESE	270.36	2.76[a]	84.01±7.04
ESL	272.37	2.69[a]	247.10±21.70
AND	286.40	2.75[a]	549.52±50.81
TES	288.41	3.31[a]	591.48±49.37
ETE	296.39	4.06±0.10	2941.33±59.74
PRO	314.45	3.84±0.05	158.84±14.25
COC	346.45	2.00±0.01	1442.12±86.89
PNS	358.44	1.46[a]	812.13±28.95
PNL	360.44	1.49±0.04	2129.53±61.51
COR	360.46	1.47[a]	1059.48±16.17
HYC	362.47	1.53[a]	1931.96±94.72
BET	392.45	2.02±0.03	991.74±87.33

a) $\log K_{o/w}$ are cited data of Hansch and Leo, 1979. *b*) Saturated concentrations Cs are measured at 37 °C. ESE; estrone, ESL; 17β-estradiol, AND; androstendione, TES; testosterone, ETE; ethinyl estradiol, PRO; progesterone, COC; corticosterone, PNS; prednisone, PNL; prednisolone, COR; cortisone, HYC; hydrocortisone, and BET; betamethasone.

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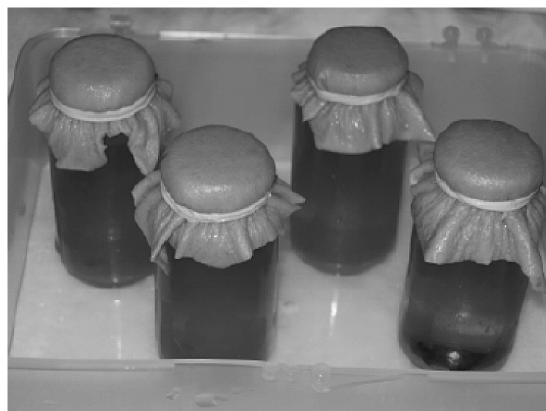

Fig. 1. Photograph of Dehydrated Skin Preparation

Dermis and stratum corneum side keep in contact with MEM medium and desiccated air, respectively. This sample keeps at 4 °C for 72 h.

vial filled with MEM medium, that is, dermis side kept in contact with MEM medium at all time (Fig. 1). After the skin was fixed by a rubber band, the sample vial was placed in a desiccator with silica gel (Sigma Chemical, Co.) and kept at 4 °C for 72 h. Thus, this procedure dehydrated only the SC and could nearly restore the hydrated skin to *in vivo* condition. Skin thickness was measured by sandwiching skin samples between plastic plate using a micrometer.[9]

Transepidermal Water Loss Measurements *in Vitro* To ensure the restoration from hydrated to dehydrated skin, transepidermal water loss (TEWL) was measured using an evaporimeter (Tewameter TM210, Courage & Khazaka, Germany) at 0, 24, 48, 72, and 96 h after skin preparation started in desiccator. Because the dehydrated skins were prepared at 4 °C, skin samples were left at room temperature for 30 min and then TEWL was measured. Measurements were performed at 23±2 °C room temperature and relative humidity of 57±3%.

***In Vitro* Skin Penetration Experiments** Skin samples were mounted between the two half cells of *in vitro* side-by-side permeation system (the effective volume is 5 ml and the effective membrane area 0.64 cm^2). The temperature in the *in vitro* system was maintained at 37 °C. A 40% PEG400 solution without chemicals (5 ml) was filled in the receptor cell to maintain sink condition. A chemical suspension in 40% PEG400 solution (5 ml) was then loaded in the donor cell. At predetermined time intervals, 300 μl samples were withdrawn from the receptor cell and assayed for the concentration of model chemical by HPLC.

Assay Methods All chemicals were assayed by HPLC (1100 series, Hewlett Packard; pump: G1311A, detector: G1314A, degasser: G1322A, and autosampler: G1313A). The column was an Luna 5u C18(2), 250×4.6 mm (phenomenex, Torrance, CA) with guard column (Security Guard, C18 4×3.0 mm, phenomenex). Other analytical conditions were summarized in Table 2.

Student's *t*-test for paired samples was used for statistical evaluation.

RESULTS

Effect of Hydration on Skin Thickness Skin thickness are 1.147±0.256 mm (stripped skin, $n=77$), 1.163±0.286 mm (dehydrated skin, $n=67$), and 1.199±0.319 mm (hydrated skin, $n=79$), respectively. SC thickness of hydrated skin (52 μm) are 3.3 times that of dehydrated skin (16 μm).

Table 2. Analytical Conditions of Model Chemicals Used

	Wavelength [nm]	Mobile phase	Flow rate [ml/min]	Retention time [min]
ESE	210	52% Acetonitrile	0.8	11.6
ESL	210	52% Acetonitrile	0.8	8.2
AND	235	60% Acetonitrile	0.8	8.7
TES	238	62% Methanol	0.8	6.5
ETE	281	72% Methanol	0.8	9.0
PRO	240	62% Methanol	0.8	8.5
COC	240	60% Methanol	0.8	10.1
PNS	240	58% Methanol	0.8	8.9
PNL	240	60% Methanol	0.8	10.4
COR	240	60% Methanol	0.8	8.9
HYC	240	60% Methanol	0.8	11.0
BET	238	65% Methanol	0.8	9.7

Abbreviations are indicated in a footnote of Table 1.

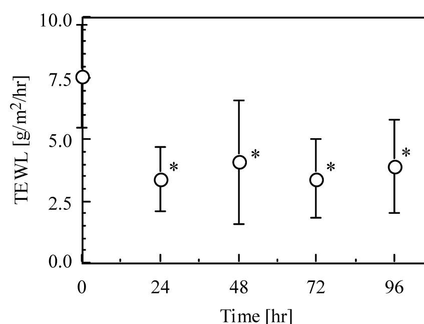

Fig. 2. Recovery of Transepidermal Water Loss (TEWL) with Time by the Dehydrated Skin Preparation Procedure (see Materials and Methods)

Significantly different from TEWL at 0 h at $p<0.05$ (*), which are calculated using *t*-test.

TEWL Measurement Time course of TEWL under the condition of dehydrated skin preparation shows in Fig. 2. TEWL of hydrated skin (0 h) is 7.6±2.3 $g/m^2/h$. The values after 24 h become a almost constant (average 3.7 $g/m^2/h$) and differ from hydrated skin, having a statistically significant difference ($p<0.05$).

Effect of Hydration on Skin Penetration Physicochemical properties of model chemicals are summarized in Table 1. Octanol/water partition coefficients ($\log K_{o/w}$) of ESE, ESL, AND, TES, PNS, COR, and HYC are referred from Hansch and Leo.[10] The ratio of steady-state penetration flux (J) and lag-time (t), x-axis of J on cumulative amount of penetrated *vs.* time plot, for hydrated skin (J_h and t_h) to dehydrated skin (J_d and t_d) is shown in Table 3. The ratio of J_h/J_d ranged between 0.7 and 3.7 (average value of 1.8). On the other hand, the ratio of t_h/t_d is almost constant (average 0.8). Liner regression of J_h/J_d and t_h/t_d against MW and log $K_{o/w}$ did not yield a good relationship ($r^2=0.001$—0.024) (Figs. 3, 4).

DISCUSSION

Permeants have different molecular weight, molecular structure, lipophilicity (solubility in skin and donor compartment), melting point, and ionization degree. These physicochemical properties affect skin permeability.[1—4] Skin ab-

Table 3. Penetration Parameters across Dehydrated and Hydrated Intact Skin

	J_i [μg/cm^2/h]			t_i [h]		
	Dehydrated skin (J_d)	Hydrated skin (J_h)	J_h/J_d	Dehydrated skin (t_d)	Hydrated skin (t_h)	t_h/t_d
ESE	0.02±0.01	0.07±0.04	3.07	7.75±1.37	5.32±2.87	0.69
ESL	0.04±0.04	0.04±0.02	0.84	8.20±4.44	6.49±1.74	0.79
AND	0.68±0.33	0.82±0.20	1.21	4.16±0.92	4.15±1.69	1.00
TES	0.24±0.07*	0.72±0.24*	2.91	4.59±1.82	6.79±0.76	1.24
ETE	0.11±0.04	0.20±0.07	1.76	8.55±1.63*	4.19±0.53*	0.49
PRO	0.27±0.12	0.25±0.06	0.93	8.02±4.45	5.12±2.34	0.51
COC	0.20±0.11	0.43±0.23	2.17	8.10±2.64	6.87±5.07	1.16
PNS	0.02±0.02	0.05±0.06	3.65	10.62±2.77**	5.02±2.00**	0.47
PNL	0.05±0.06	0.04±0.02	0.74	9.09±1.06	13.57±1.13	1.49
COR	0.03±0.04	0.05±0.01	1.61	5.67±1.80	4.93±3.05	0.87
HYC	0.03±0.02	0.07±0.06	2.26	7.04±4.89	3.94±1.76	0.56
BET	0.002±0.001	0.002±0.001	1.00	25.12±2.39	19.93±7.35	0.79

Abbreviations are indicated in a footnote of Table 1. Statistically significant differences between dehydrated and hydrated skin at $p<0.05$ (*) and $p<0.1$ (**) calculated using *t*-test are noted.

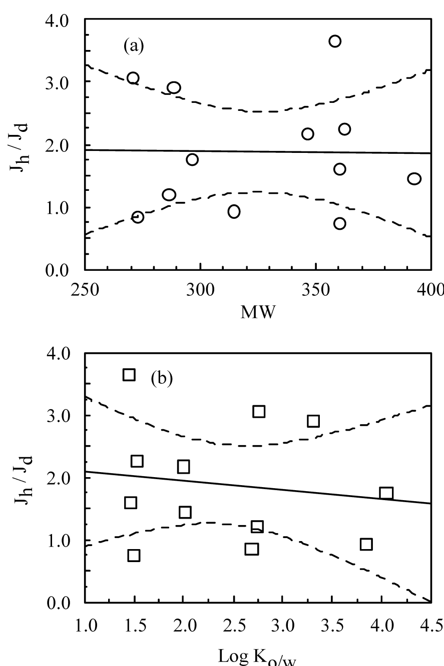

Fig. 3. Relationship between the Ratio of J_h/J_d and MW (a) and log $K_{o/w}$ (b)

Steady-state flux across hydrated skin J_h and dehydrated skin J_d are obtained from *in vitro* penetration experiments under same condition. Solid and dashed lines represent a regression line and 95% confidence intervals, respectively.

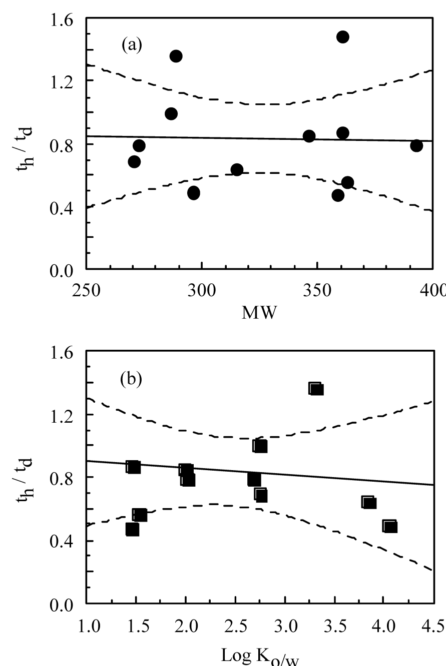

Fig. 4. Relationship between the Ratio of t_h/t_d and MW (a) and log $K_{o/w}$ (b)

Lag-times (t) of hydrated skin t_h and dehydrated skin h_d are calculated from x-axis of J on cumulative amount of penetrated *vs.* time plot. Solid and dashed lines represent a regression line and 95% confidence intervals, respectively.

sorption is also influences by skin condition; water contents (hydration) of SC[11] and skin surface pH.[5] Researchers have discussed the influence of physicochemical properties of drugs on percutaneous absorption *in vitro* and *in vivo*[8] and reported the effect of hydration and *in vitro* experimental conditions (pH and composition of vehicle). We selected twelve model chemicals having a similar steroidal structure but a different molecular weight (270.4 to 392.5) and log $K_{o/w}$ (1.46 to 4.06) to investigative the effect of hydration on skin penetration flux J and lag-time t under the same experimental conditions *in vitro*.

TEWL, a water permeability of skin, has a correlation with percutaneous absorption flux.[12] Thus, integrity of SC barrier function was investigated by a measurement of TEWL. Water overlaid on the SC of hydrated skin was evaporated and the water vapor lasted for 10 min at least.[13] The skin samples were left at room temperature more than 30 min for TEWL was accurately measured in this study. TEWL of hydrated skin (7.6±2.1 g/m^2/h) is obviously larger than that of dehydrated skin (24 h; 3.4±2.1 g/m^2/h, 48 h; 4.1±2.5 g/m^2/h, 72 h; 3.4±1.6 g/m^2/h, and 96 h; 3.9±1.9 g/m^2/h, Fig. 2) which are almost same values with back (4.51±0.57 g/m^2/h) and leg (4.39±0.32 g/m^2/h) skin *in vivo* data.[14] Thus, SC barrier is recovered by the procedure of dehydrated skin preparation for 24 h and over.

Hydration of SC increases J and slightly decreases t through the skin (Table 3). The ratio of J_h/J_d and t_h/t_d had no relation to MW, a range between 270.4 to 392.5, and log $K_{o/w}$ from 1.5 to 4.1 (Figs. 3, 4). This result may be caused by a narrow range of MW and log $K_{o/w}$. Thus, we will eventually

confirm a relation between a wide range of physicochemical properties and skin penetration parameters.

The SC is composed of dehydrated-flat cells (hydrophilic domains) in hydrophobic lipid domains. Intercellular lipids are arranged in a dense and orderly bilayer and influence skin barrier function.[15] Most lipophilic permeants permeates across these lipid domains. One mechanism of penetration enhancers is to disrupt the arranged lipid bilayers and enhance skin permeability.[16,17] The dried and flat corneocytes in SC absorb water. SC thickness of hydrated skin was 3.3 times thicker than that of dehydrated skin in this study. The swollen SC increases its weight to 300 to 400 times and water are mainly absorbed within the corneocytes[18] resulting in disrupted lipid bilayers.[19,20] This is a reason why SC barrier function decreased with increasing contact time with water. On the other hand, the SC barrier function caused by lipid bilayers might return to its *original* state (Fig. 2) because we prepared the dehydrated skin from the hydrated skin under a mild condition (at 4 °C and for 72 h). We used 40% PEG400 solution to maintain sink conditions. This solution rapidly decreased skin water content, mainly viable epidermis and dermis, from 68% (wt/wt) to 24% (wt/wt) for a few hours.[21] This rapid change of the water content in the skin may keep disordering lipid bilayers in hydrated skin.

Skin samples obtained from a skin bank for research were generally preserved in culture medium until use for *in vitro* penetration experiments. Therefore, we consider the possibility that J was overestimated and t was underestimated using hydrated skin. We confirmed the *in vitro* difference of J and t between hydrated and dehydrated skin and will define these results under *in vivo* conditions.

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