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Effect of fatty acids on the transdermal delivery of donepezil: *In vitro* and *in vivo* evaluation

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ABSTRACT

The effect of fatty acids on the skin permeation of donepezil base (DPB) and its hydrochloride salt (DPH) were studied *in vitro* using hairless mouse and human cadaver skin. DPB and DPH were solubilized in propylene glycol (PG) containing 1% (w/v) fatty acid, after which the *in vitro* permeation through hairless mouse skin and human cadaver skin were evaluated using Keshary-Chien diffusion cells. The optimized formulation obtained from the *in vitro* study was then tested in rats for an *in vivo* pharmacokinetic study. The relative *in vitro* skin permeation rate of donepezil (DP) through the hairless mouse skin showed a parabolic relationship with increased carbon length of the fatty acid enhancers. Among the fatty acids tested, oleic acid for DPB and palmitoleic acid for DPH showed the highest enhancing effect, respectively. Both the permeation rates of DPB and DPH evaluated in human cadaver skin were in good correlation with those in hairless mouse skin, regardless of the presence of fatty acids. This suggests that the mouse skin model serves as a useful *in vitro* system that satisfactorily represents the characteristics of the human skin. Moreover, based on the *in vitro* results, the optimal formulation that could maintain the human plasma concentration of 50 ng/mL was determined to be 10 mg DP with 1% (w/v) enhancer. When the DP transdermal formulations were applied to the abdominal skin of rats (2.14 cm^2), the C_{ss} was maintained for 48 h, among which the highest value of 52.21 ± 2.09 ng/mL was achieved with the DPB formulation using oleic acid. These results showed that fatty acids could enhance the transdermal delivery of DP and suggested the feasibility of developing a novel transdermal delivery system for clinical use.

1. Introduction

Alzheimer's disease is a chronic neuro-degenerative disorder that causes a progressive decline in cognitive function, deficits in activity of daily living and behavioral disturbances (Terry and Buccafusco, 2003). Alzheimer's disease is the most common cause of dementia in elderly people. As the number of persons affected by Alzheimer's disease is growing enormously owing to the rapid growth of older-generation populations, Alzheimer's disease related problems are one of the most important medical, social and economic problems in the contemporary society (Brookmeyer et al., 1998).

Although the exact pathogenic mechanism of Alzheimer's disease is not fully understood as yet, it is generally accepted that deficit resulting from the impairment of cholinergic neurotransmission is responsible for the symptoms (Bartus et al., 1982; Drachman and Leavitt, 1974). Thus, cholinesterase inhibitors were the first category of drugs approved for use in Alzheimer's disease and include tacrine, donepezil (DP), rivastigmine, and galantamine (Seltzer, 2007). However, the large fluctuations in plasma concentration levels after oral administration of these drugs have been associated with high incidence of gastrointestinal adverse effects including diarrhea, nausea, and vomiting (Imbimbo, 2001; Jann et al., 2002).

A transdermal drug delivery system can be considered as an alternative dosage form for cholinesterase inhibitors because it could provide an efficacious drug level in systemic circulation through a sustained drug delivery, while reducing the incidences of adverse events by avoiding the large fluctuation of plasma concentration (Winblad and Machado, 2008). Moreover, since dementia makes self medication practically very difficult, it would be helpful to apply a transdermal patch type of formulation with prolonged drug action for these Alzheimer's disease patients. These patches would improve patient compliance as well as provide the advantage of visibility of the drug to the caregiver (Blesa et al., 2007; Volicer et al., 1987). These transdermal delivery patches have been developed for rivastigmine and became available in many countries since 2007 (Grossberg et al., 2009; Winblad and Machado, 2008). Clinical trials have shown that the effect of the rivastigmine transdermal patch was comparable to that of

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the rivastigmine capsule, and rates of nausea and vomiting were lowered.

DP, a piperidine-based reversible acetylcholinesterase inhibitor, is currently the most prescribed pharmacological agent for the treatment of Alzheimer's disease. DP is superior to other acetylcholinesterase inhibitors due to its high potency and selectivity for the enzyme in the central nervous system (Heydorn, 1997). Since DP also causes gastrointestinal adverse effects, a transdermal delivery system might be a suitable method to overcome the problems of conventional oral administration of DP.

Thus, the main objective of the present study was to develop a transdermal delivery system for DP. Because the therapeutic efficacy of a drug for transdermal delivery mainly depends on its ability to penetrate the skin high enough to provide the effective plasma concentrations to present the desired pharmacological activity (Paolo et al., 2001), fatty acids were selected as a skin permeation enhancer for improving the transdermal permeation of DP. Fatty acids have been successfully used as penetration enhancers for several drugs (Blesa et al., 2007; Green et al., 1988; Ogiso and Shintani, 1990). For this, we systematically investigated the effects of various fatty acids on the permeation of DP using hairless mouse skin, and compared the *in vitro* permeability of DP in hairless mouse and human cadaver skin. Finally, *in vivo* pharmacokinetic studies were performed to assess the feasibility of its transdermal delivery.

2. Materials and methods

2.1. Materials

DP base (DPB) and its hydrochloride salt (DPH) were received as a gift from Chong Kun Dang Pharm. Co. Ltd. (Seoul, Korea). The fatty acids listed in Table 1 were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and were of the highest available purity. Phosphate buffered saline (PBS, pH 7.4, 20 mM; Hyclone) and acetonitrile were obtained from Thermo Fisher Scientific Co. (Pittsburgh, PA, USA). Monobasic potassium phosphate was purchased from Duksan Pure Chemical Co., Ltd. (Ansan, Korea). Sodium hydroxide solution (1 M) and propylene glycol (PG) were obtained from Samchun Pure Chemical Co., Ltd. (Pyeongtaek, Korea). Methanol and ethyl acetate were of analytical grade from Aldrich Chemical Co. (Milwaukee, WI, USA).

2.2. Solubility of DPB and DPH in PG with various fatty acids

The solubility of DPB and DPH in PG with various fatty acids (1%, w/v) was determined at 37 °C. Excess amount of DPB or DPH was added to PG, and mixed by vortexing. The suspended solution was shaken in a water bath at 37 °C for 24 h to reach equilibrium. The equilibrated sample was centrifuged at 13,200 rpm for 5 min to remove the undissolved DPB or DPH. The supernatant was taken and was analyzed by HPLC after dilution with the mobile phase.

2.3. Animals

Male hairless mice weighing 18–20 g (Orient Bio Inc., Sungnam, Korea) were used in *in vitro* permeation experiments, while male Sprague–Dawley rats weighing 250–300 g (Orient Bio Inc.) were used in *in vivo* pharmacokinetics experiments. The animals were fasted overnight before dosing, with free access to food and water. Experimental protocols for the animals used in this study were reviewed by the Animal Care and Use Committee of the College of Pharmacy, Seoul National University, according to the National Institute of Health guidelines (National Institutes of Health Publication Number 85-23, revised 1985) in Principles of Laboratory Animal Care.

2.4. In vitro skin permeation study of DP

In vitro skin permeation study across hairless mouse skin or human cadaver skin was conducted with Keshary-Chien diffusion cells at 37 °C. The hairless mice were sacrificed by cervical dislocation right before the experiments. The dorsal skin was cut into appropriate sizes after carefully removing subcutaneous fat, and then the skin was clamped between the donor and the receptor cells with the stratum corneum side facing upward into the donor. The human cadaver skin (Hans Biomed, Seoul, Korea) stored in a freezer (−18 °C) was used after thawing in warm normal saline for 10 min before the permeation studies. The donor cells (1.0 mL), which contained various concentrations (1, 5, 10 mg/mL, and saturated) of DPB or DPH in PG with or without 1–5% (w/v) fatty acids, were occluded with parafilm. The receptor cells were filled with PBS (pH 7.4, 20 mM). The area of diffusion cell for all *in vitro* studies was 2.14 cm^2. The diffusion cell was maintained at 37 °C using a water bath and the solution in the receptor chambers was stirred continuously at 600 rpm. At predetermined time intervals (2, 4, 6, 8, 10, and 12 h), 1.0 mL of the solution in the receptor cell was withdrawn, and replaced immediately with an equal volume of fresh receptor media. Concentrations of DP were analyzed by HPLC.

2.5. HPLC analysis of DP

The concentration of DP was analyzed by an HPLC system, consisting of a UV detector (Waters 2487 Dual λ Absorbance Detector, Milford, MA, USA), a pump (Waters 515 HPLC Pump), and an automatic injector (Waters 717 plus Auto-sampler). The wavelength of the UV detector was set at 268 nm. A reversed-phase column (CAPCELL PAK, 5 μm, 250 mm × 4 mm, Shiseido, Japan) was used as the stationary phase at ambient temperature. The mobile phase consisted of phosphate buffer (0.01 M KH_2PO_4) and acetonitrile (65:35, v/v), and was eluted at a flow rate of 1.0 mL/min.

2.6. In vivo pharmacokinetic study of DP

Abdominal hair of rats was removed with an electric clipper, and depilatory cream was applied to the skin. After one day, they

Table 1
Fatty acid analogues evaluated as skin permeation enhancers.

Enhancer	Name	Formula	Melting point (°C)	Remark
C12:0	Lauric acid (dodecanoic acid)	$CH_3(CH_2)_{10}COOH$	44.2	Saturated
C14:0	Myristic acid (tetradecanoic acid)	$CH_3(CH_2)_{12}COOH$	58.8	Saturated
C16:0	Palmitic acid (hexadecanoic acid)	$CH_3(CH_2)_{14}COOH$	63.1	Saturated
C16:1	Palmitoleic acid (cis-8-hexadecenoic acid)	$CH_3(CH_2)_5CH{=}CH(CH_2)_7COOH$	−0.5	Unsaturated
C18:0	Stearic acid (N-octadecanoic acid)	$CH_3(CH_2)_{15}COOH$	69.6	Saturated
C18:1	Oleic acid (cis-9-octadecenoic acid)	$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$	13.4	Unsaturated
C18:2	Linoleic acid (cis-9,12-octadecadienoic acid)	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$	−5	Unsaturated
C18:3	Linolenic acid (cis-9,12,15-octadecatrienoic acid)	$CH_3CH_2CH{=}CH\ CH_2CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$	−11	Unsaturated
C20:0	Arachidic acid (N-eicosanoic acid)	$CH_3(CH_2)_{18}COOH$	76.5	Saturated

were anesthetized with ketamine (Yuhan Co., Ltd., Seoul, Korea, dose: 50 mg/kg, *im*) and acepromazine (Samwoo Chemical Co., Ltd., Seoul, Korea, dose: 10 mg/kg, *im*). After confirming the induction of anesthesia, they were fixed in supine position and the femoral artery (for blood sampling) was cannulated with a PE-50 polyethylene tube (Clay Adams, Parsippany, NJ, USA) filled with heparinized saline (20 IU/mL, for preventing blood clotting). After recovery from the anesthesia, DPB or DPB in PG (10 mg/mL) with or without 1% (w/v) fatty acids were applied to the abdomen of the rats at a dose of 40 mg/kg. For transdermal application to the rats, a specially designed device with a diffusion area of 2.14 cm^2 was mount on the abdomen and fixed with surgical glue (Vetbond®, 3M Co., St. Paul, MN, USA), as reported in the literature (Valiveti et al., 2004) which made it possible to apply DP in PG solution without loss of liquid. Blood samples were collected from the femoral artery cannula at predetermined time intervals after DP administration. The blood withdrawn at each time point was replaced immediately with an equal volume of normal saline to compensate for fluid loss. The plasma fractions were obtained from the centrifugation of blood samples at 13,200 rpm for 5 min. The plasma samples were stored at −80 °C until used for the LC–MS analysis of DP.

2.7. LC–MS analysis of DP

A liquid chromatography–mass spectrometry (LC–MS) method was used to determine the concentration of DP in the plasma samples using diphenhydramine as an internal standard (IS) (Xie et al., 2006). To a 100 μL aliquot of plasma sample, 10 μL of diphenhydramine solution (10 μg/mL in methanol) and 100 μL of phosphate buffer (10 mmol/L, pH 14) were added. The tubes were vortexed briefly and ethyl acetate (1000 μL) was added. This resulting mixture was vortexed for 30 min and then centrifuged at 13,200 rpm for 5 min. The supernatant was then transferred to a fresh tube and evaporated to dryness under a gentle stream of N_2 gas at room temperature. Each dried residue was redissolved in 100 μL of acetonitrile, vortexed for 10 min, and transferred to the LC–MS autosampler vials.

The HPLC system consisted of a Waters 2695 separation module (quaternary pump and autoinjector) and a Synergi 4μ MAX-RP column (75 mm × 4.6 mm, 4 μm; Phenomenex, Torrance, CA, USA). The isocratic mobile phase, consisting of 90:10 (v/v) mixture of solvent A (50% acetonitrile in methanol) and solvent B (0.01 M ammonium acetate, pH 4), was pumped at 0.4 mL/min. Column elute was analyzed with a Waters ZQ (Milford, MA, USA) single quadrupole mass spectrometer, equipped with an electronspray probe interfaced to a liquid chromatograph, operating in electronspray, positive-single-ion mode to monitor 380 *m*/*z* for DP and 256 *m*/*z* for IS. The response of the detector was linear in the concentration range examined (*i.e.*, 0.1–500 ng/mL) for the plasma samples with inter-/intra-day precisions of less than 15% and an accuracy within 15% of the theoretical value, indicating the assay was valid in the range of concentration studied.

2.8. Pharmacokinetic analysis

The pharmacokinetic analysis of plasma concentrations–time profiles of DP after transdermal administration was carried out by using the WinNolin software (version 4.0, Pharsight Co., Mountain View, CA, USA). Transdermal delivery data of DP were analyzed using a non-compartmental model to determine peak concentration (C_{max}), lag time to steady-state concentration (T_{lag}), and area under curve from 0 to 48 h (AUC_{0-48}). Finally, the C_{ss} of DP for 48 h were calculated by using the equation:

$$C_{ss} = \frac{AUC_{0-t}}{t} \quad (1)$$

where t is 48 h.

2.9. Statistical analysis

All experiments were performed at least three times (n = 3–5) and were expressed in the form of the mean ± S.D. For comparison of mean values between the experimental groups, the Student's t-test was carried out. In all cases, $p < 0.05$ was accepted as denoting a statistical difference.

3. Results

3.1. Solubility study

The saturated solubility of DP base (DPB) and its hydrochloride salt form (DPH) in PG without fatty acid was 18.11 ± 0.57, 139.00 ± 3.64 mg/mL, respectively. However, the corresponding saturated solubility of DPB and DPH in PG was not affected by the addition of fatty acids (Tables 2 and 3).

3.2. Effect of fatty acids on the skin permeation of DP through hairless mouse skin

Saturated solutions of DP were used for *in vitro* permeation studies to maintain maximum thermodynamic activity of drug in the donor compartment throughout the studies, although the saturated solubility of DP was significantly different between the base and the salt form. In case of DPB, stearic acid (C18) increased the permeation rate of DP most effectively among the saturated fatty acids of 12–20 carbon units (Table 2). That is, the permeation rate of DP with 1% (w/v) of stearic acid was 15.55 μg/h/cm^2, which was a 9.54-fold increase compared to that of DP without a permeation enhancer (1.63 μg/h/cm^2, Table 2). Although the permeation rate of the DPH showed similar values among the saturated fatty acids of 12–20 carbon units, palmitic acid (C16) seemed to be the most

Table 2
In vitro hairless mouse skin permeation parameters of DP when DPB was saturated in PG containing 1% (w/v) of fatty acid at 37 °C.

Enhancer	Permeation rate (μg/h/cm^2)	Permeability coefficient (cm/h) × 10^{-3}	Lag time (h)	Solubility (mg/mL)	ER[a]
None	1.63 ± 0.51	0.09 ± 0.02	5.30 ± .151	18.11 ± 0.57	1.00
C12:0	1.30 ± 1.05	0.10 ± 0.07	3.52 ± 1.46	18.59 ± 1.23	0.80
C14:0	2.98 ± 0.36[†]	0.15 ± 0.02	3.45 ± 0.81	20.39 ± 1.38	1.83
C16:0	1.88 ± 0.50	0.09 ± 0.02	3.89 ± 0.51	20.36 ± 1.42	1.15
C18:0	15.55 ± 1.92[†]	0.75 ± 0.11	4.82 ± 0.86	20.71 ± 0.40	9.54
C20:0	11.50 ± 1.81[†]	0.60 ± 0.19	3.78 ± 0.84	19.57 ± 2.61	7.05
C18:1	19.31 ± 2.06[†]	0.95 ± 0.11	5.48 ± 0.27	20.43 ± 0.63	11.85
C18:2	9.84 ± 1.30[†]	0.46 ± 0.05	4.24 ± 0.28	21.52 ± 0.82	6.04
C18:3	3.84 ± 0.20[†]	0.18 ± 0.01	3.19 ± 1.15	21.97 ± 1.37	2.36

Each data represents the mean ± S.D. (n = 3).
[a] ER indicates the enhancing ratio: ER = $\frac{\text{permeation rate in the presence of enhancer}}{\text{permeation rate without enhancer}}$.
[†] Significantly different ($p < 0.05$) from without enhancer.

Table 3
In vitro hairless mouse skin permeation parameters of DP when DPH was saturated in PG containing 1% (w/v) of fatty acid at 37 °C.

Enhancer	Permeation rate (μg/h/cm^2)	Permeability coefficient (cm/h) × 10^{-3}	Lag time (h)	Solubility (mg/mL)	ER[a]
None	0.90 ± 0.13	0.0065 ± 0.0011	6.63 ± 0.51	139.00 ± 3.64	1.00
C12:0	1.32 ± 0.45	0.0098 ± 0.0035	3.34 ± 1.67	136.00 ± 9.46	1.47
C14:0	2.34 ± 1.54	0.0158 ± 0.0091	5.48 ± 1.26	143.60 ± 14.55	2.60
C16:0	2.80 ± 0.63[†]	0.0199 ± 0.0052	5.16 ± 0.19	141.22 ± 6.27	3.11
C18:0	1.07 ± 0.15	0.0078 ± 0.0008	2.71 ± 0.50	136.99 ± 6.14	1.19
C20:0	1.47 ± 0.08[†]	0.0106 ± 0.0004	5.08 ± 0.32	138.40 ± 1.66	1.63
C16:1	98.29 ± 5.53[†]	0.7217 ± 0.0336	7.53 ± 0.09	136.29 ± 7.86	104.88

Each data represents the mean ± S.D. (n = 3).
[a] ER indicates the enhancing ratio: ER = $\frac{\text{permeation rate in the presence of enhancer}}{\text{permeation rate without enhancer}}$.
[†] Significantly different (p < 0.05) from without enhancer.

effective in enhancing the skin permeation of DP (Table 3). Since the solubility of DPB and DPH in PG was not affected by the addition of the saturated fatty acids, the increase in the permeation rate seems to be resulted from the increase in the permeability coefficient (Tables 2 and 3). On the basis of the above results, stearic acid (C18) and palmitic acid (C16) were selected for DPB and DPH, respectively, for the evaluation of enhancing effect of corresponding unsaturated fatty acid.

The addition of the unsaturated fatty acids significantly changed the permeation properties of DPB compared with the presence of the corresponding saturated fatty acid (Fig. 1). A parabolic relationship was observed between the permeation rate and number of double bond in fatty acid. Oleic acid (C18:1) slightly increased the permeation rate in case of DPB. However, as the number of double bonds increased from linoleic acid (C18:2) to linolenic acid (C18:3), a substantial decrease in the permeation rate was observed (Fig. 1A). On the other hand, in case of DPH, the permeation rate was 35-fold enhanced with the addition of palmitoleic acid (C16:1), compared to that in palmitic acid (Table 3, Fig. 1B). Thus, oleic acid and palmitoleic acid was selected as permeation enhancers for DPB and DPH, respectively, for further studies.

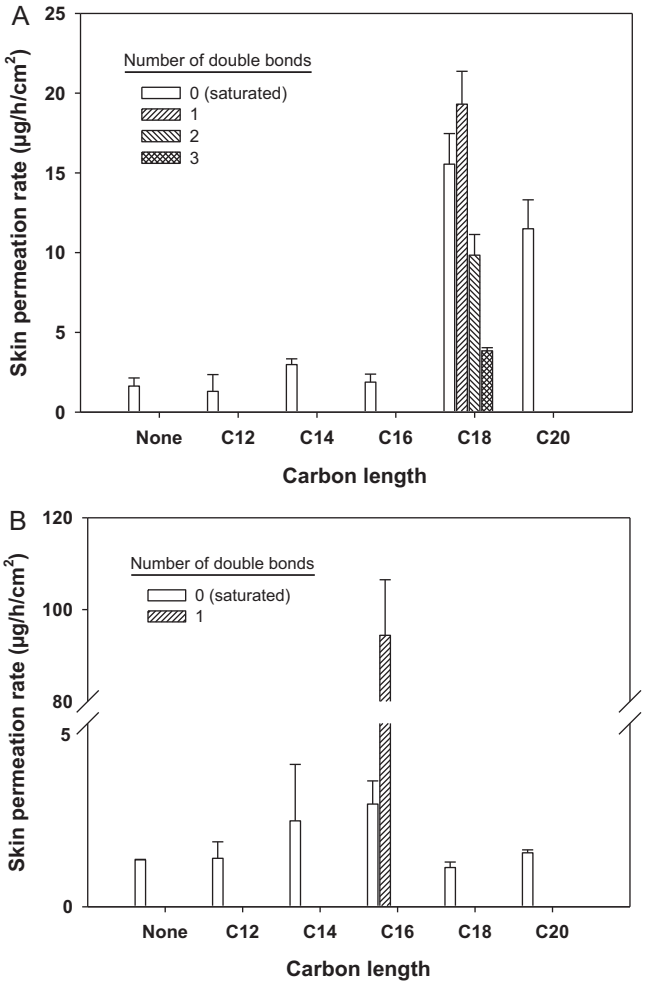

Fig. 1. Effect of various fatty acids on the skin permeation rate of DP when DPB (A) or DPH (B) was saturated in PG containing 1% (w/v) of fatty acid at 37 °C. Each point represents the mean ± S.D. (n = 3).

3.3. Effect of concentration of DP and fatty acid on skin permeation of DP through hairless mouse skin

To develop the optimal formulations of DP for the clinical application, the effect of concentrations of DP and fatty acid on the skin permeation was investigated. In case of DPB without enhancer, the permeation rate linearly increased from 0.41 μg/h/cm^2 to 3.90 μg/h/cm^2, as the concentration of drug in the vehicle increased from 1 mg to 10 mg (Table 4). In case of DPH without enhancer, however, skin permeation rate was significantly low (0.14–0.19 μg/h/cm^2) and did not increase in a loading dose-dependent manner over the range 1–10 mg, which is probably due to the negligibly low loading dose compared to its saturated solubility in PG (136.29 mg/mL, Table 4).

The addition of fatty acid (1%, w/v) in PG vehicle enhanced the skin permeation rate of DP in both DPB and DPH cases over the loading concentration range of 1–10 mg (Table 4). In DPB, the permeation rates of DP (1, 5, and 10 mg) in the presence of oleic acid (1%, w/v) were 5–8 folds higher (2.02–31.14 μg/h/cm^2) than those of DPB alone (0.41–3.90 μg/h/cm^2). However, the permeation rate of DP at the loading dose of 10 mg DPH with the addition of 1% (w/v) of palmitoleic acid was only 5.21 μg/h/cm^2, indicating that 1% (w/v) of enhancer is not enough to increase the skin permeation rate of DPH. In addition, the effect of different concentration of fatty acids (1, 2, and 5%) on the permeation of DP is shown in Table 4. An increasing trend in permeation rate of DP was observed with the increase in concentration of fatty acids. The permeation rate of DP with 5 mg of DPB and 5% oleic acid (21.94 μg/h/cm^2) increased by 12.3-fold compared with that of DPB alone (1.79 μg/h/cm^2). Moreover, it is interesting to note that the addition of 5% (w/v) palmitoleic acid did not further increase the skin permeation rate of DP compared to that of 2% (w/v), indicating that around 14 μg/h/cm^2 is the maximum permeation rate that can be achieved by the 5 mg dose when palmitoleic acid was used as a permeation enhancer. Yet, 5% palmitoleic acid in 5 mg DPH loading dose increased the permeation rate of DP (14.22 μg/h/cm^2) by 79-fold compared with that of DPH without enhancer (0.18 μg/h/cm^2), which value is considerably higher than that of the 10 mg dose of DPH with 1% palmitoleic acid (5.21 μg/h/cm^2).

It has been reported that there was a statistically significant positive correlation between plasma concentrations of DP and acetylcholinesterase inhibition (Machado and Caramelli, 2006;

Table 4
The effect of DP concentration and fatty acid on skin permeation of DP in hairless mouse skin *in vitro*.

Drug (mg/mL in PG)		Enhancer % (w/v)		Permeation rate ($\mu g/h/cm^2$)	Permeability coefficient ($cm/h) \times 10^{-3}$	Lag time (h)	Remarks
Donepezil base (DPB)	1	Oleic acid	0	0.41 ± 0.08	0.41 ± 0.08	3.88 ± 0.64	
	1		1	2.02 ± 0.44	2.02 ± 0.44	4.93 ± 0.22	
	5		0	1.79 ± 0.20	0.36 ± 0.04	4.18 ± 0.57	
	5		1	13.00 ± 1.17	2.60 ± 0.23	5.62 ± 0.37	
	5		2	14.96 ± 1.86	2.99 ± 0.37	4.95 ± 0.79	
	5		5	21.94 ± 7.24	4.39 ± 1.45	5.03 ± 0.45	
	10		0	3.90 ± 0.21	0.39 ± 0.02	4.52 ± 0.18	Rx 1
	10		1	31.14 ± 7.18	3.11 ± 0.72	5.97 ± 0.45	Rx 2
Donepezil HCl (DPH)	1	Palmitoleic acid	0	0.14 ± 0.01	0.14 ± 0.01	3.17 ± 0.52	
	1		1	0.82 ± 0.11	0.82 ± 0.11	5.55 ± 0.46	
	5		0	0.18 ± 0.02	0.04 ± 0.004	3.18 ± 0.50	
	5		1	4.50 ± 0.86	0.90 ± 0.17	6.74 ± 0.45	
	5		2	14.09 ± 3.02	2.82 ± 0.60	6.76 ± 0.49	
	5		5	14.22 ± 3.16	2.84 ± 0.63	6.19 ± 0.45	
	10		0	0.19 ± 0.03	0.02 ± 0.003	3.90 ± 1.05	Rx 3
	10		1	5.21 ± 0.77	0.52 ± 0.08	6.59 ± 0.70	Rx 4

Each data represents the mean ± S.D. ($n = 3$).

Rogers et al., 1998b; Schneider et al., 1998). The EC_{50} of DP in human was reported to be 15.6 ng/mL and a maximum inhibition was obtained at plasma concentrations of higher than 50 ng/mL (Rogers et al., 1998a). The target permeation rate of DP to maintain the steady-state plasma concentration (C_{ss}, ng/mL) higher than the therapeutic level after transdermal application can be calculated from the following equation:

$$\text{Permeation rate} = \frac{C_{ss} \times CL_t \times BW}{A} \quad (2)$$

where CL_t, A and BW represent the total body clearance, the surface area of transdermal delivery device and the body weight of the subject, respectively. Therefore, from Eq. (2), the target permeation rate can be estimated to be 16.5 $\mu g/h/cm^2$ to maintain the steady-state plasma concentration of DP over 50 ng/mL, assuming that a transdermal delivery device which has a surface area of 20 cm^2 is applied to a patient with a body weight of 60 kg (Kim and Chien, 1996). The CL_t (0.11 L/h kg) in human was obtained from the literature (Tiseo et al., 1998). Among the test formulations shown in Table 4, two formulations consists of 5 mg DPB with 5% oleic acid (21.94 $\mu g/h/cm^2$) and 10 mg DPB with 1% oleic acid (31.14 $\mu g/h/cm^2$) satisfied the permeation rate criteria. However, a skin irritation problem caused by skin enhancers should be considered in selecting a higher concentration of the skin enhancer (Kanikkannan and Singh, 2002). Thus, the formulation of 10 mg DPB with 1% oleic acid (Rx 2) was selected for further evaluation, and the formulation of 10 mg DP without enhancer (Rx 1) was used for comparison, together with 10 mg DPH formulations (Rx 3 and 4).

3.4. Comparison of DP permeabilities between hairless mouse skin and human cadaver skin

To evaluate a correlation between the skin permeability of DP in mouse and human, *in vitro* permeation study of DPB and DPH through human cadaver skin were conducted using Rx 1, 2, 3, and 4 formulations. The relevant permeation parameters in human cadaver skin study are summarized in Table 5. Also, the predicted C_{ss} at each formulation was calculated from Eq. (2), and is shown in Table 5. The permeation rate and permeability coefficient in human cadaver skin were significantly lower than that in the hairless mouse skin for test formulations, whereas the lag times to steady-state permeation rate were similar for both human and hairless mouse (Tables 4 and 5). Although it is well known that rodent skins are generally more permeable than human skin (Scott et al., 1986), the difference in permeation rate and permeability coefficient were less than 2.8-times between human skin and hairless mouse skin in all formulations tested. It is notable that the predicted steady-state plasma concentration (C_{ss}) of DP was a 48.95 ng/mL in the Rx 2 formulation, which was similar to the target plasma concentration in human (*i.e.*, 50 ng/mL).

3.5. In vivo pharmacokinetic studies in rats

To evaluate the feasibility of transdermal delivery of DP, *in vivo* pharmacokinetic studies were performed in rats after transdermal application of four different formulations of DP. The plasma concentration–time profiles of DP are shown in Fig. 2. The relevant pharmacokinetic parameters, C_{max}, T_{max}, C_{ss}, $AUC_{0-48\,h}$, and T_{lag} are given in Table 6. The values of C_{ss} and $AUC_{0-48\,h}$ were significantly higher in Rx 2 and Rx 4 compared with Rx 1 and Rx 3, respectively, which were consistent with results from the *in vitro* permeation study using the hairless mouse and human cadaver skin. As a result, C_{max} and $AUC_{0-48\,h}$ in Rx 2 were 67.2 ng/mL and 2505.9 ng h/mL, respectively. Interestingly, the steady-state levels were maintained for more than the 48 h of the application period in all formulation groups which indicated that sufficient driving force for transdermal DP delivery was maintained for more than 48 h after application. Moreover, the steady-state plasma concentration (C_{ss}) of DP in the

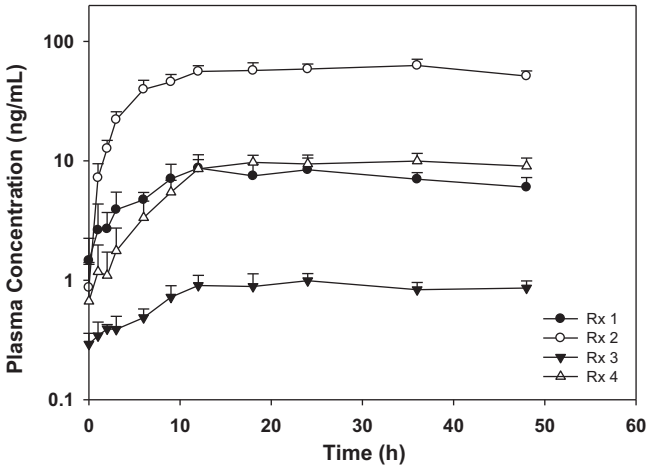

Fig. 2. Plasma concentration profiles of DP after applying DPB or DPH to the abdomen of the rats (2.14 cm^2) at a dose of 40 mg/kg. DPB or DPB was solubilized in PG at 10 mg/mL with or without 1% (w/v) fatty acids. Each point represents the mean ± S.D. ($n = 5$). Rx 1: DPB alone; Rx 2: DPB with oleic acid; Rx 3: DPH alone; Rx 4: DPH with palmitoleic acid.

Table 5
In vitro human cadaver skin permeation parameters of DP 10 mg in PG containing 1% (w/v) of fatty acids at 37 °C.

Drug (mg/mL in PG)		Enhancer % (w/v)		Permeation rate ($\mu g/h/cm^2$)	Permeability coefficient $(cm/h) \times 10^{-3}$	Lag time (h)	Predicted C_{ss} in human (ng/mL)	Remarks
Donepezil base (DPB)	10	Oleic acid	0	1.41 ± 0.26	0.14 ± 0.03	5.20 ± 0.57	4.26 ± 0.77	Rx 1
	10		1	16.15 ± 1.36†	1.62 ± 0.14†	6.45 ± 0.54	48.95 ± 4.13	Rx 2
Donepezil HCl (DPH)	10	Palmitoleic acid	0	0.14 ± 0.03	0.01 ± 0.003	4.64 ± 1.09	0.42 ± 0.08	Rx 3
	10		1	2.06 ± 0.23†	0.21 ± 0.02†	6.48 ± 0.35	6.26 ± 0.71	Rx 4

Each data represents the mean ± S.D. ($n = 3$).
† Significantly different ($p < 0.05$) from Rx1 or Rx3 for Rx2 and Rx4, respectively.

Table 6
Pharmacokinetic parameters of DP with DPB or DPH solubilized in PG with or without 1% (w/v) fatty acid applied to the rat abdomen at a dose of 40 mg/kg.

Parameters	Donepezil base (DPB)		Donepezil HCl (DPH)	
	Rx 1	Rx 2	Rx 3	Rx 4
C_{max} (ng/mL)	9.70 ± 1.76	67.20 ± 6.22†	1.09 ± 0.12	10.49 ± 1.17†
T_{max} (h)	19.2 ± 6.6	24.0 ± 11.2	30.0 ± 16.9	20.4 ± 9.1
T_{lag} (h)	7.8 ± 2.7	9.6 ± 2.5	10.8 ± 1.6	13.2 ± 2.7
AUC_{0-48} (ng·h/mL)	329.25 ± 60.40	2505.86 ± 152.23†	38.77 ± 4.46	387.70 ± 49.40†
C_{ss} (ng/mL)	6.86 ± 1.26	52.21 ± 2.09†	0.81 ± 0.09	8.08 ± 1.03†

Each data represents the mean ± S.D. ($n = 5$).
† Significantly different ($p < 0.05$) from Rx1 or Rx3 for Rx2 and Rx4, respectively.

Rx 2 formulation was 52.21 ng/mL, which was close to the target and the predicted C_{ss} in human (48.85 ng/mL, Table 5).

4. Discussion

Since human skin is designed to protect an organism from the external environment and is effective as a barrier to chemical transport, the skin penetration of drug is an important issue for clinical relevance of transdermal delivery system (Paolo et al., 2001). It has been reported that skin penetration and absorption are influenced by the physicochemical properties of a drug, including lipophilicity, molecular weight, size, and structure (Bos and Meinardi, 2000; Nitti et al., 2006). As a small (*i.e.* molecular weight of 379.5 (Thevis et al., 2006)) and lipophilic molecule (*i.e.* Log *P* value of 3.08–4.11 (Xia et al., 2008)), DP is considered to be physicochemically well-suited for transdermal delivery. In our preliminary study using aqueous solution formulations, the permeation rates of DP across hairless mouse skin were not high enough to reach the target plasma levels (data not shown), and thus PG was selected as a vehicle in this study. However, since the salt form would be too hydrophilic for transdermal delivery, the base form was anticipated to give better permeability. As expected, the solubility of DPH in PG was significantly higher than the DPB while permeability was much higher in the latter compared to the salt form resulting in the higher skin permeation rate of DPB at the same loading concentration.

In the present study, fatty acid was used as a permeation enhancer to increase the skin penetration of DP. Fatty acids are the most attractive skin permeation enhancers used for transdermal drug delivery system and also commonly used as an adjuvant in cosmetics and pharmaceuticals because most fatty acids are endogenous components of the human skin (Santoyo and Ygartua, 2000; Stott et al., 2001; Tanojo et al., 1997). In this study, mono-unsaturated fatty acid exhibited an effective permeation-enhancing effect in the skin permeation of DP compared to the corresponding saturated fatty acid. As a result, the permeation rate of DP increased up to 19.31 $\mu g/h/cm^2$ and 98.29 $\mu g/h/cm^2$, when DPB or DPH was saturated in PG with 1% (w/v) oleic acid and palmitoleic acid, respectively. Because the solubility of DP in PG was not significantly affected by the addition of the fatty acids, the increase in the permeation rate seems to be related to the increase in the permeability coefficient. Therefore, these results might be explained by the formation of a "kink" isomer at the double bond. That is, the *cis*-configuration of unsaturated fatty acids alters the phase structure of the intercellular lipid matrix and makes free volumes (Harrison et al., 1996). This small and mobile free volume in the skin could increase the fluidity permitting small molecules to enter the free volumes of the kinks and migrate across the membrane (Träuble, 1971). Considering that the kinks tend to increase with the number of double bonds in the unsaturated fatty acids, the skin permeation of DP is expected to increase with the increase of the number of double bond in the fatty acid. However, the skin permeation of DP significantly decreased as the number of double bond increased in this study (Fig. 1A), which seemed to be related with steric hindrance, limiting the partitioning of the fatty acid to the lipid bilayers of the stratum corneum (Kim et al., 2008).

Although species difference has been reported in skin properties such as the difference in the stratum corneum thickness (Behl Cr et al., 1994) and the composition of skin lipids (Wertz and Downing, 1989) which have a direct effect on the skin permeability of drug, animal skins play a crucial role in the development of a transdermal delivery system. In this study, the difference in permeation parameter between human and hairless mouse skin were below 2.8-fold in all formulations tested. No significant difference was observed in the lag time between hairless mouse and human skin. Moreover, the same rank order in the skin permeation was observed in both skin types. As a result, a linear relationship in *in vitro* permeation rate between human cadaver skin and hairless mouse skin was shown regardless of the DP form and the presence of fatty acid ($y = 0.5285x - 0.9925$, $r^2 = 0.9897$) (Fig. 3), indicating the usefulness of hairless mouse skin for predicting the *in vitro* permeation rate of DP through human skin.

Since transdermal delivery is a multistep process of the drug release from the formulation applied to the skin surface and its transport to the systemic circulation (Guy and Hadgraft, 1986), *in vivo* study was needed to evaluate the pharmacokinetic properties. The *in vivo* study was conducted in rats because pharmacokinetic study is not technically possible in mice. Rodent skin has disadvantages of extremely high density of hair follicles compared with that of human skin. The risk of injury to cutaneous tissue as hair removal may affect percutaneous permeation of drugs. Despite these limitations, rodent models are commonly used in *in vivo* transdermal delivery studies because of their small size, uncomplicated handling and relatively low cost (Godin and Touitou, 2007). Among rodents, rats have been reported

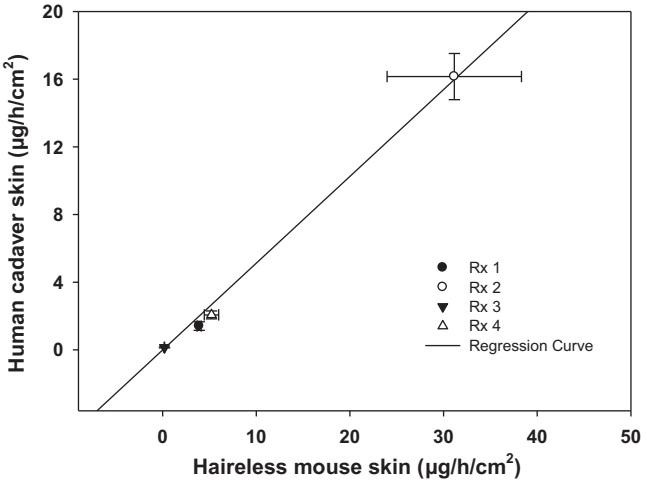

Fig. 3. Relationship of the *in vitro* skin permeation rate of DP between human cadaver skin and hairless mouse skin. DPB or DPB was solubilized in PG at 10 mg/mL with or without 1% (w/v) fatty acids. Each point represents the mean ± S.D. ($n = 5$). Rx 1: DPB alone; Rx 2: DPB with oleic acid; Rx 3: DPH alone; Rx 4: DPH with palmitoleic acid.

to represent structural similarities to human tissue in terms of thickness of stratum corneum, epidermis, and whole skin (Ronald and Howard, 2005). Moreover, transappendageal absorption, relative to transepidermal diffusion through the stratum corneum is of limited importance in the overall process of percutaneous absorption during steady-state conditions (Kao et al., 1988). When administered to rats, as shown in Fig. 2, plasma concentration of DP reached a mean steady-state level at around 8 h which was maintained for at least 48 h in all formulations, which is a promising result for possible clinical formulation development.

5. Conclusions

The permeation enhancement of DP by fatty acids was systemically evaluated using *in vitro* and *in vivo* methods. In *in vitro* permeation study, a parabolic relationship between the permeation enhancement and carbon-chain lengths of fatty acids was obtained, with oleic acid for DPB and palmitoleic acid for DPH showing the highest effect. The permeation rate of DP through human cadaver skin was in good correlation with that through hairless mouse skin regardless of the presence of fatty acids, suggesting that *in vitro* permeation study using hairless mouse skin can be used for predicting human skin permeability of DP. Considering the target permeation rate in human, DP formulation consisting of 10 mg of DPB with 1% oleic acid seems to be the optimum in maintaining the required therapeutic plasma levels for future clinical transdermal formulations of DP.

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