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# Pharmacological evaluation and safety of a donepezil patch

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Our aim was to assess the feasibility of transdermal delivery of donepezil and evaluate the pharmacokinetics (PK), pharmacodynamics (PD), and safety of donepezil patch *in vitro* and *in vivo*. Donepezil patches were applied to the skin of rabbits and humans for 7 days, then, the PK profiles were observed in a dose-dependent manner. Donepezil was continuously released from the patch for 7 days as compared to oral administration in hairless rats and rabbits. In hairless rats, peak acetylcholinesterase (AChE) inhibition of 34.7±2.0% was observed within 8 h after oral administration of 4 mg/head donepezil, and lasted for less than 24 h, consistent with changes in the plasma donepezil concentration. Peak AChE inhibition by the donepezil patch was equivalent to that in the orally administered group. Donepezil was released continuously from the patch for 7 days with a linear PK in both rats and rabbits. AChE activity inhibition was dependent on donepezil plasma concentration. The data exhibited excellent PK/PD correlation. There was no dermal irritation (erythema/edema) in placebo or donepezil patch group during the study period in minipigs. Thus, Dong-A's donepezil patch appeared to be generally safe and was well tolerated.

## 1. Introduction

Alzheimer's disease (AD), a progressive neurologic disorder, is the most common cause of senile dementia. It is characterized by loss of memory and other intellectual abilities, which can interfere with the daily life of patients (Small et al. 1997). The symptoms of AD are related to a cholinergic deficit. Moreover, the extent of its neuropathologic character has been found to correlate with cholinergic loss in the central nervous system (Davis et al. 1995, Rogers et al. 1998a).

As acetylcholinesterase (AChE) is predominantly present in the brain, striated muscles, and blood, AD treatment strategies are primarily directed toward its inhibition (Sozio et al. 2012).

Donepezil ($C_{24}H_{29}NO_3$, MW: 379.492) is a reversible and selective AChE inhibitor. Donepezil can inhibit the breakdown of neurotransmitter acetylcholine (ACh) and compensate for its deficiency in the brain (Colovic et al. 2013). Furthermore, clinical trials have shown that donepezil improves the cognitive function in patients with mild-to-moderate-to-severe AD (Rogers et al. 1998a). Most common adverse events (AEs) associated with donepezil are nausea, vomiting, diarrhea, and dizziness, all of which are linked to cholinergic hyperstimulation (Rogers et al. 1998b; Burns et al. 1999; Kim et al. 2015). These effects are dose-related and largely dependent on plasma fluctuations (Imbimbo 2001).

Transdermal drug delivery systems have several advantages compared to oral preparations and injection, including improved systemic bioavailability, evasion of hepatic first-pass metabolism, lower administration frequency, longer duration of action, reduced side effects, and excellent patient compliance (Lee et al. 2016). Switching to transdermal patch from oral or injection preparations could be an effective option to treat AD (Oertel et al. 2007). A transdermal patch can reduce plasma fluctuations by maintaining sustained therapeutic plasma concentrations. In addition, it could reduce the maximum concentration ($C_{max}$) and delay the time required to reach the maximum concentration ($T_{max}$), thus decreasing the incidence of cholinergic side effects (Saluja et al. 2013).

Donepezil patch by Dong-A, a new formulation patch under development, has the advantage of smooth and steady delivery of donepezil through the skin.

The present study investigated the transdermal delivery of Dong-A's donepezil patch through human and rabbit skin and evaluated the correlation between its pharmacokinetic (PK)/pharmacodynamic (PD) characteristics and topical safety.

## 2. Investigations and results

### *2.1. In vitro drug release study*

Figure 1 shows the cumulative permeation profiles of donepezil from the transdermal delivery system. Figure 1A shows the permeated amount from rabbit and human skin plotted against time, whereas Fig. 1B shows the utilization of the drug in rabbit and human skin against time. In this study, drug utilization was assessed by calculating the transdermal permeation of the loaded drug dose in the patch. Donepezil permeated through rabbit and human skin over a period of 7 days. Approximately 68.1±6.0% of donepezil in the patch permeated transdermally through the human skin for 7 days, whereas approximately 51.2±18.6% of donepezil permeated through the rabbit skin.

### *2.2. Pharmacokinetic and pharmacodynamic profiles of donepezil patch in hairless rats*

After application of a donepezil patch to hairless rats, plasma donepezil concentration was measured up to 168 h. Figure 2A shows the plasma donepezil concentration following its transdermal administration. Table 1 lists the calculated PK profiles. After the

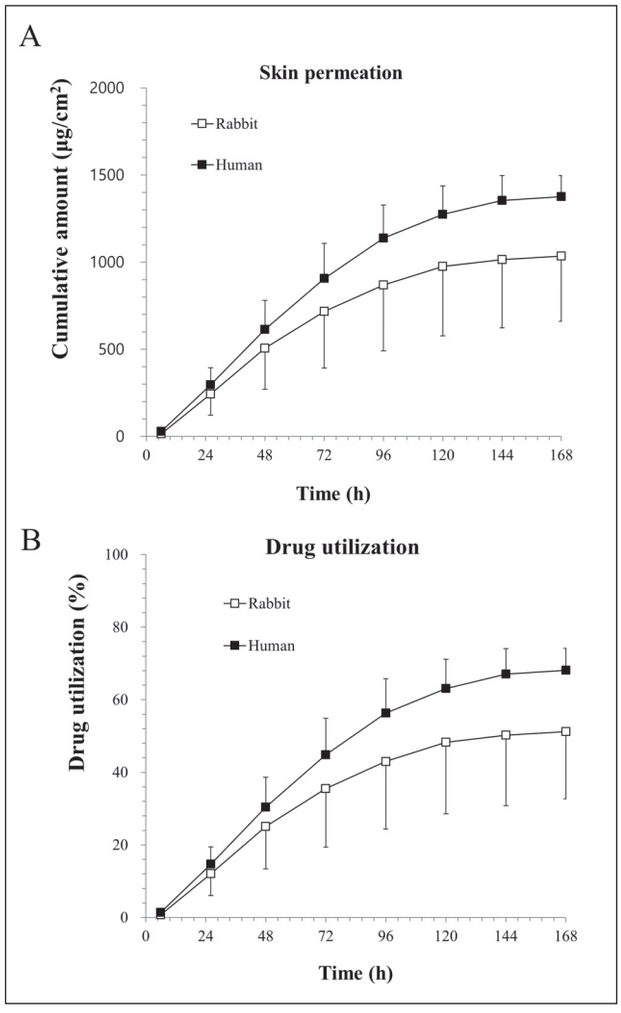


Fig. 1: (A) Cumulative amount and (B) drug utilization of donepezil for 7 days in diffusion cells. Data are expressed as mean ± standard deviation (N = 6).

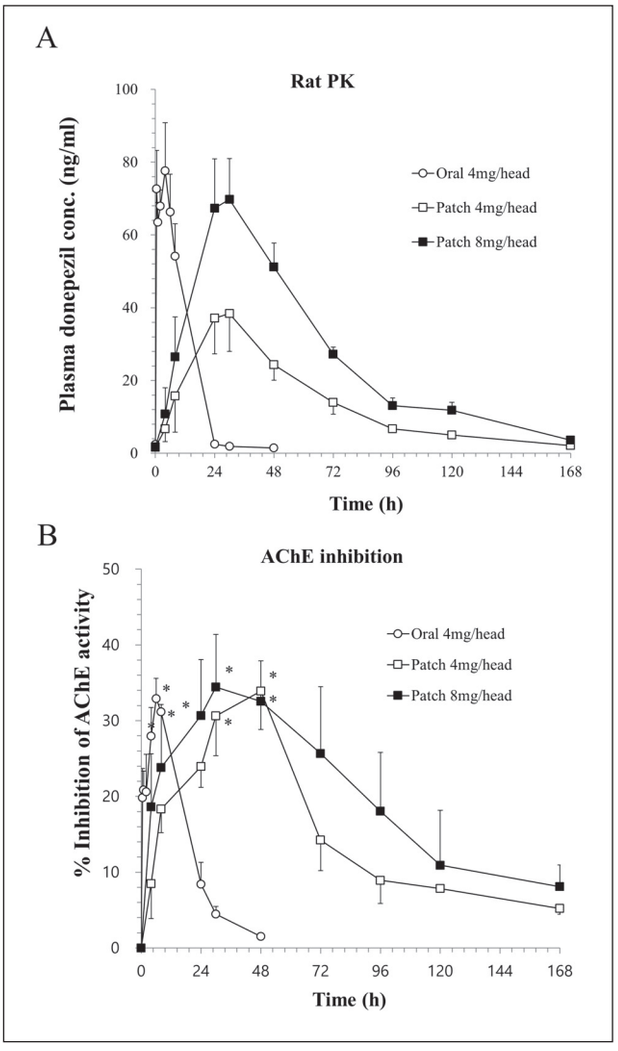


Fig. 2: Pharmacokinetic and pharmacodynamic profiles of donepezil in hairless rats. (**A**) Plasma concentration–time curve and (**B**) plasma concentration–% inhibition of AChE activity for 7 days after patch application. Data are expressed as mean ± standard deviation (N = 4–7). Abbreviations: AChE, acetylcholinesterase; n, number of subjects.

application of donepezil patch, the PK profiles were observed in a dose-dependent manner. After transdermal treatment with 4 and 8 mg/patches, the concentration reached a maximum value ($C_{max}$) at approximately 27.6±3.3 h and 31.2±9.9 h, respectively, and subsequently decreased gradually (Fig. 2A). For the 4 mg/patch, the $C_{max}$ and area under the curve (AUC) were 39.0 ±9.9 ng/mL and 2034.6 ± 505.7 ng*h/mL, respectively. For the 8 mg/patch, these values were 70.1±11.5 ng/mL and 3776.3±622.9 ng*h/mL, respectively. A bolus oral treatment at 4 mg/head was administered to hairless rats (Fig. 2A) and $C_{max}$ and AUC were 81.2±8.1 ng/mL and 733.6±187.3 ng*h/mL, respectively (Table 1).

Acetylcholinesterase (AChE) enzyme activity was measured with oral donepezil and donepezil patches. Peak AChE inhibition of 34.7±4.4% was observed within 8 h after oral administration and it lasted less than 24 h in the 4.0 mg/head group (Fig. 2B), consistent with the changes in the plasma donepezil concentration. The peak AChE inhibition by the donepezil patch was equivalent to that in the oral group and was observed within 40 h after application at a dose of 4.0 or 8.0 mg (Table 2), coinciding with the plasma $T_{max}$. The mean maximal AChE inhibition by the patch ($E_{max}$: 36–38%) was comparable between 4.0 and 8.0 mg dose groups despite the fact that there was a dose-proportional increase in the mean $C_{max}$ from 39.0 to 70.1 ng/mL (Fig. 2B).

**Table 1: Donepezil pharmacokinetic parameters after oral or patch application to hairless rats**

| Parameter | PO | Donepezil patch | |
|---|---|---|---|
| Dose | 4 mg/head | 4 mg/head | 8 mg/head |
| $T_{max}$ (h) | 2.6 ± 1.7 | 27.6 ± 3.3 | 31.2 ± 9.9 |
| $C_{max}$ (ng/mL) | 81.2 ± 8.1 | 39.0 ± 9.9 | 70.1 ± 11.5 |
| $AUC_{0\rightarrow last}$ (ng*h/mL) | 733.6 ± 187.3 | 2034.6 ± 505.7 | 3776.3 ± 622.9 |

After transdermal treatment with (4 and 8) mg/patch, the $C_{max}$ and AUC were observed in a dose-dependent manner. Notes: Data are expressed as mean ± standard deviation (N = 4-7).
Abbreviation: PO, per oral; $T_{max}$, the time taken to reach the maximum concentration; Cmax, the maximum (or peak) plasma concentration; AUC, area under curve.

**Table 2: AChE inhibitory activity effects after oral or patch application in hairless rats**

| Parameter | PO | Donepezil patch | |
|---|---|---|---|
| Dose | 4 mg/head | 4 mg/head | 8 mg/head |
| Time to peak (h) | 7.2 ± 1.1 | 37.2 ± 9.9 | 32.4 ± 9.1 |
| Peak effect (%) | 34.7 ± 4.4 | 36.9 ± 11.3 | 36.6 ± 12.1 |
| $AUC_{0\rightarrow last}$ (%h) | 387.8 ± 214.0 | 1993.9 ± 749.8 | 2681.4 ± 1652.6 |

AChE enzyme activity was measured with donepezil oral and patches. Peak AChE inhibition of (34.7±4.4) % was observed within 8 h after oral administration. Peak AChE inhibition of the donepezil patch (4 mg/head and 8 mg/head) was equivalent to that in the oral group. Notes: Data are expressed as mean ± standard deviation (N = 4-7).
Abbreviation: PO, per oral; AChE, acetylcholinesterase; AUC, area under curve.

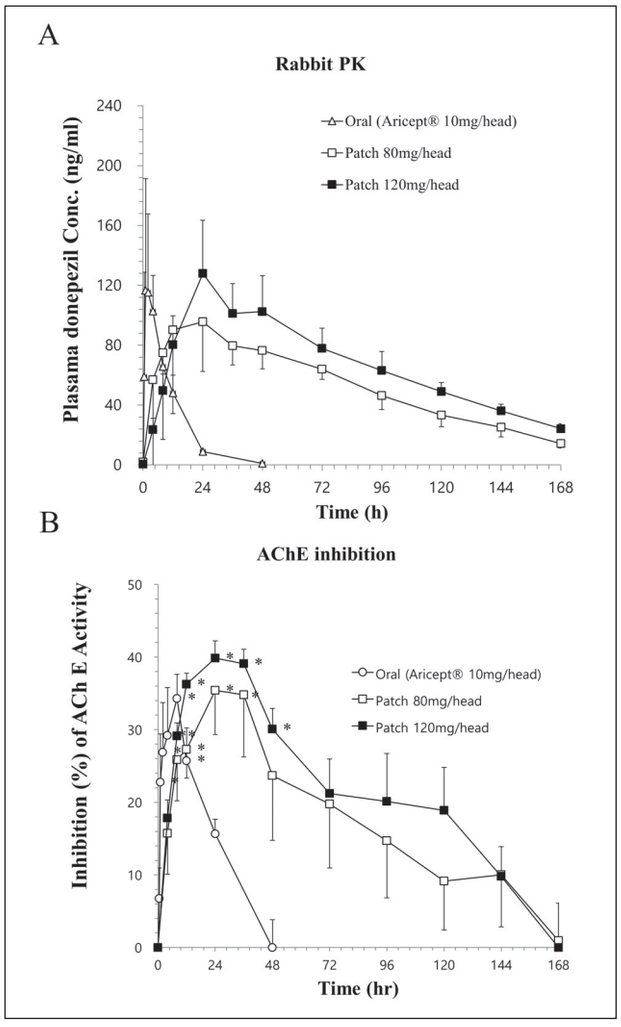


Fig. 3: Pharmacokinetic and pharmacodynamic profiles of donepezil in NZW rabbits. (**A**) Plasma concentration–time curve and (**B**) plasma concentration–% inhibition of AChE activity for 7 days after patch application. Data are expressed as mean ± standard deviation (N = 5). Abbreviations: NZW, New Zealand White; n, number of subjects.

### *2.3. Pharmacokinetic and pharmacodynamic profiles of donepezil patch in NZW rabbits*

After treatment of NZW rabbits with Aricept® or donepezil patch, plasma donepezil concentration was measured. The concentration of donepezil was measured from 0 to 48 h after oral administration of Aricept® at 10 mg/head. Figure 3A shows the plasma donepezil concentration following oral administration. Table 3 lists the calculated PK profiles.

**Table 3: Donepezil pharmacokinetic parameters after oral or patch application to NZW rabbit**

| Parameter | Aricept® PO | Donepezil patch | |
|---|---|---|---|
| Dose | 10 mg/head | 80 mg/head | 120 mg/head |
| $T_{max}$ (h) | 2.8 ± 0.6 | 28.8 ± 24.9 | 28.8 ± 10.7 |
| $C_{max}$ (ng/mL) | 131.7 ± 56.7 | 113.7 ± 33.0 | 130.4 ± 33.7 |
| $AUC_{0\rightarrow inf}$ (ng*h/mL) | 1319.8 ± 190.4 | 9859.2 ± 1569.6 | 13084.3 ± 1259.2 |
| Normalized BA (%) | – | 74.7% | 99.1% |

After oral treatment with Aricept® at 10 mg/head or donepezil patch (80 mg/head and 120 ng/head), PK profiles was calculated. The normalized bioavailability of patch was calculated to be 74.7 ~ 99.1%. Notes: Data are expressed as mean ± standard deviation (N = 5).
Abbreviations: PO, per oral; $T_{max}$, the time taken to reach the maximum concentration; $C_{max}$, the maximum (or peak) plasma concentration; AUC, area under curve; BA, bioavailability.

After oral treatment with Aricept® at 10 mg/head as a suspension, $C_{max}$ was achieved after approximately 2.8±0.6 h and rapidly decreased (Fig. 3A). The $C_{max}$ and AUC values were 131.7±56.7 ng/mL and 1319.8 ± 190.4 ng*h/mL, respectively. After application of the donepezil patch, PK profiles were observed in a dose-dependent manner. After transdermal treatment at 80 and 120 mg/patch, $C_{max}$ was reached after approximately 28.8±24.9 h and 28.8±10.7 h, respectively, and subsequently, the concentration decreased gradually (Fig. 3A). The $C_{max}$ and AUC were 113.7±33.0 ng/mL and 9859.2±1569.6 ng*h/mL for the 80 mg/patch group and 130.4±33.7 ng/mL and 13084.3±1259.2 ng*h/mL, respectively, for the 120 mg/patch group. The normalized bioavailability of patch was calculated to be 74.7 to 99.1%, indicating that the donepezil could slowly permeate the patch transdermally. It had the same level of exposure as the oral Aricept® at 10 mg.

The AChE activity was measured following treatment with oral donepezil and donepezil patches. Peak AChE inhibition of 36.5±9.8% was observed within 8 h after oral administration, which lasted less than 24 h in the 10 mg/head group (Fig. 3B), consistent with the changes in the plasma donepezil concentration. The peak AChE inhibition of the donepezil patch was equivalent to that in the oral group and was observed within 30 h after application of an 80 or 120 mg dose (Table 4).

**Table 4: Inhibitory AChE activity effects after oral or patch application in NZW rabbit**

| Parameter | Aricept® PO | Donepezil patch | |
|---|---|---|---|
| Dose | 10 mg/head | 80 mg/head | 120 mg/head |
| Time to peak (h) | 6.0 ± 2.8 | 28.0 ± 6.6 | 24.0 ± 8.5 |
| Peak effect (%) | 36.5 ± 9.8 | 38.9 ± 15.3 | 41.9 ± 3.1 |
| $AUC_{0\rightarrow last}$ (%h) | 702.0 ± 362.3 | 3187.1 ± 1999.7 | 3559.8 ± 1126.5 |

AChE enzyme activity was measured with donepezil oral and patches in NWZ rabbits. Peak AChE inhibition of the donepezil patch was equivalent to that in the oral group. **Notes**: Data are expressed as mean ± standard deviation (N = 5).
Abbreviations: PO, per oral; AChE, acetylcholinesterase; AUC, area under curve.

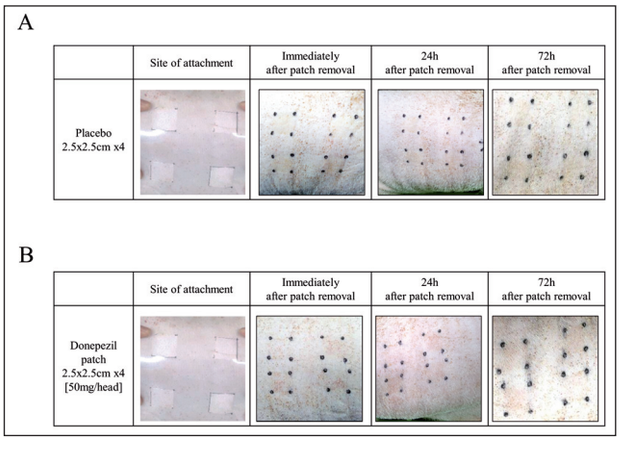


Fig. 4: Topical skin irritation in minipig. (A) Placebo patch and (B) donepezil patch after application for 7 days.

### *2.4. Topical skin tolerance to donepezil patch in minipig*

A 7-day study was conducted in male minipig to characterize dermal irritation by the donepezil patch. Prior to dosing, eight application sites on each minipig (approximately 2.5 cm × 2.5 cm each on left side, donepezil patch; right side, placebo) were marked with an indelible marker. Seven days after application, there was no dermal irritation (erythema/edema) in the male minipig to whom a placebo (Fig. 4A) or donepezil patch (Fig. 4B) had been applied topically. The primary irritation index (PII) calculated by scores of readings was 0 in both the placebo and donepezil patch groups (Table 5). This suggested that the PII category of the donepezil patch was negligible.

**Table 5: Primary index scores after placebo or donepezil patch application to minipig**

| Group | PII score | Classification |
|---|---|---|
| Placebo | 0 | Non-irritating |
| Donepezil patch | 0 | Non-irritating |

A 7-day dermal irritation study was conducted in male minipig to characterize dermal irritation of the donepezil patch. At 7 days after application, there were no dermal irritations (erythema/edema) in male minipig applied topically with placebo or donepezil patch
Abbreviations: PII, primary irritation index.

## 3. Discussion

In the present study, we characterized the PK, PD, and safety of Dong-A's donepezil patch both *in vitro* and *in vivo*.

Our data indicate that donezepil from Dong-A's donepezil patch could permeate through rabbit and human skin over a period of 7 days *in vitro* with linear PK profiles *in vivo*. Furthermore, we demonstrated that the donepezil patch was generally well tolerated. The capacity of a drug to penetrate the skin is an important aspect for clinical relevance of transdermal delivery systems. The efficacy and the rate of absorption of a drug through the skin depend on several biologic and physiochemical factors (Sozio et al. 2012). The present study evaluated the diffusion of donepezil into human or rabbit skin tissue using Franz cell setup to predict the permeation in humans and animals. Dong-A's donepezil patch continuously permeated through the skin for 7 days and approximately 50 to 70% of the dose was released from the patch over the 7-day application. These data suggest that donepezil patch could be used for1 week for continuous release of donepezil.

Transdermal donepezil delivery is advantageous in reducing the dosing frequency owing to its longer duration of action while improving the bioavailability and maintaining plasma levels, thereby resulting in reduced fluctuation of plasma levels, compared to the oral route of administration (Sozio et al. 2012). After application in rats, the donepezil patch gradually increased, with sustained plasma concentration–time profiles over the patch-on period, compared to that on oral treatment. The donepezil patch demonstrated linear PK characteristics.

AChE activity is a sensitive biomarker that is positively correlated with donepezil plasma concentration. AChE inhibition depends on plasma donepezil concentration, and reflects improvement in cognitive and global functions (Rogers et al. 1998a). To measure the PD response to AChE inhibition, an AChE activity assay was conducted using rat plasma. Although inhibition of AChE activity depends on donepezil concentration, there is variation in the basal activity of AChE in rat plasma, which is likely to affect the maximum % inhibition (Shin et al. 2018). In the current study, plasma AChE activity was measured prior to drug administration and was regarded as basal activity (Rogers and Friedhoff 1998; Ito et al. 2010). The maximum inhibition achieved after donepezil treatment was approximately 30 to 40%, consistent with previous reports (Shin et al. 2018). Inhibition of AChE was positively correlated with donepezil concentrations in the plasma. In the current study, donepezil did not produce complete inhibition of AChE in the rat plasma, which was similar to the results of other studies (Haug et al. 2005). These data suggest that donepezil could not completely inhibit rat plasma cholinesterase because the cholinesterase consists of both AChE and butyrylcholinesterase (Traina and Serpietri 1984; Kosasa et al. 2000). Previous clinical literature reports the intra-subject circadian variability in AChE activity to be 2.6% (Rogers and Friedhoff 1998). Moreover, daily fluctuation in the plasma AChE activity in female rats was reported. However, there was no daily fluctuation in the plasma AChE activity in male rats (Alves-Amaral et al. 2010). In the present study, we used male hairless rats; therefore, we supposed that there would be no circadian variation effect on the AChE activity.

Rabbits were selected as experimental models in the current study because they are easily available. They have been reported to be the most suitable animal model candidates owing to their closest permeability characteristics to the human skin, after pigs and monkeys (Bartek et al. 1972; Godin and Touitou 2007). The PK study of donepezil patch performed in rabbits showed prolongation of $T_{max}$ after patch application. Donepezil was continuously released from the patch for 7 days compared to that by oral treatment. Pharmacokinetic analysis of the donepezil patch suggested that its peak plasma concentration and AUC increased with increased drug dose. Normalized systemic exposure after 7 days application of an 80–120 mg/patch was similar to that of Aricept® at 10 mg. These data suggested that if the patch size increased, it was possible to obtain $C_{max}$ equivalent to that of oral Aricept® 10 mg, because the skin permeability data using Franz cells showed that donepezil continuously permeated transdermally for 7 days through rabbit skin.

Minipig is a well-recognized animal model for dermal toxicology. It is known that the skin of minipig is considerably similar to that of humans (Stricker-Krongrad et al. 2017). Moreover, minipig's skin surface characteristics, thickness, layers, pigmentation, turnover kinetics, number of hairs and hair follicles, blood flow, and variations by gender, age, and body region are generally similar to those in humans. Close anatomic, biochemical, and physiologic similarities of minipig skin to the human skin make minipigs a favorable non-rodent species in the efficacy and safety assessment of dermal topical products. Minipigs have been widely used by pharmaceutical companies for non-clinical skin irritation studies. The main disadvantages of transdermal delivery include potential skin irritation (Sozio et al. 2012). Therefore, skin irritation by the donepezil patch was evaluated in the minipig. After 7 days of application, there were no dermal irritations (erythema/edema) in the placebo or donepezil patch groups. This suggested that the PII category of Dong-A's donepezil patch was negligible.

Taken together, our findings indicate that Dong-A's donepezil patch showed linear PK profiles and excellent PK/PD correlation and appeared to be generally well tolerated; however, randomized controlled studies are required to further define the characteristics of this patch.

## 4. Experimental

### *4.1. Materials*

Donepezil base was obtained from Neuland Laboratories (Telangana, Hyderabad, India). Aricept® was obtained from Eisai Korea (Seoul, Korea). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise noted.

### *4.2. Ethics statement*

All animal studies were approved by the Institutional Animal Care and Use Committee of the Dong-A ST Research Institute (IACUC approval nos. I-1710231 and I-1804098). Male, hairless, 6-week-old rats and 10-week-old rabbits were obtained from the Central Lab. Animal Inc. (Seoul, Korea) and Raon-Bio (Yongin, Gyeonggi, Korea), respectively. Male minipigs with an average weight of 20 kg were obtained from Medi Kinetics (Pyeongtaek, Gyeonggi, Korea). Animals were acclimated for a week. Animals were maintained at 23±2 °C with a 12h:12h light–dark cycle (lights on 07:00–19:00) and were provided with access to food and water *ad libitum*.

### *4.3. Patch preparation*

The donepezil patch used in the study was manufactured by the Research Institute of Dong-A ST. As the patch prepared by using the salt form of the drug showed extremely low permeability, it was produced using donepezil base. The drug mixture was obtained by dissolving styrene–isoprene–styrene block copolymer and terpene resin in ethyl acetate. Next, the donepezil base was added and mixed properly. The mixture was subsequently cast on a release liner coated with silicone (Knife Coating Device; Kipae, Suwon, Gyeonggi, Korea). The solvent was removed by evaporation at 60 °C for 30 min in a dryer (LDO-080N; Daihan Labtech, Namyangju, Gyeonggi, Korea). The dried adhesive layer was laminated onto the backing film.

### *4.4. Measurement of in vitro skin permeation rate*

Skin permeation rates of donepezil patch were measured using Diffusion Cell Drive Console™ of Labfine (FCDV-15; Anyang, Gyeonggi, Korea). Permeation experiments were performed using isolated New Zealand White (NZW) rabbit skin or human skin. The diffusion cell temperature was maintained at 32 °C using a heating system with control by a keypad and a light emitting diode (LED) display. The surface area of the receiver cell opening was 0.64 $cm^2$ and its volume was 5.0 mL. Skin was excised from the NZW rabbit that was sacrificed with diethyl ether. The subcutaneous fat was removed with scissors and a scalpel. Human skin was supplied by Hans Biomed (HuSKIN™; Seoul, Korea). The receiver cell was filled with buffer solution (10% ethanol in saline) and the medium was stirred using a magnetic bar at 600 rpm.

A donepezil patch (2.0 mg/cm$^2$) was placed on the stratum corneum, and the excised skin was mounted onto each receiver cell. O-ring and the cell top were placed on the top of each skin and were subsequently clamped. Samples were collected for 168 h and quantitatively analyzed by high-performance liquid chromatography (HPLC) (Thermo Fisher Scientific, Donierstr, Germany). An Inertsil® C18 (ODS-2) column (150 mm × 4.6 mm internal diameter; 5 µm packing, GL science, Tokyo, Japan) was used with UV detection at 271 nm. The mobile phase consisted of 650:350:1 deionized water:acetonitrile:perchloric acid at a flow rate of 1 mL/min. The column temperature was maintained at 35 °C and the injection volume was 10 µL.

### *4.5. Pharmacokinetic and pharmacodynamic study in hairless rat*

To assess the PK/PD correlation, hairless rats with an average weight of 350 g were randomly assigned to corresponding groups of 4 to 7 animals per group. The hairless rats were anesthetized with diethyl ester, patched, and dressing with adhesive stretch bandages for patch fixation. Donepezil (4 mg/2 cm$^2$/head or 8 mg/4 cm$^2$/head) was applied to the back skin of hairless rats. Blood (250 µL) was collected from the tail vein. Sampling was continued until 168 h at each indicated time point of $t$ = 0, 4, 8, 24, 30, 48, 72, 96, 120, and 168 h. Samples were kept at -80 °C until analysis of donepezil concentration or AChE activity. Donepezil (4 mg/head ≅ 11.7 mg/kg) was administered orally and the blood (250 µL) was collected from the tail vein. Sampling was continued until 48 h at each indicated time point of $t$ = 0, 0.5, 1, 2, 4, 6, 8, 24, 30, and 48 h. Samples were kept at -80 °C. AChE activity was measured using a colorimetric enzyme-linked immunosorbent assay (ELISA) kit from Abcam (Cambridge, MA, USA) according to the manufacturer's instructions. In this study, AChE activity at $t$ = 0 was defined as 100% basal activity, and the AChE activity after donepezil treatment was measured to calculate AChE inhibition % versus basal activity.

### *4.6. Pharmacokinetic and pharmacodynamic study in NZW rabbits*

NZW rabbits with an average weight of 2.5 kg were assigned randomly to the corresponding groups (5 animals per group). Twenty-four hours before the patch treatment, the back skin was shaved with an electric hair clipper without damaging the skin. Donepezil patch (80 mg/40 cm$^2$/head or 120 mg/60 cm$^2$/head) was applied at the back skin of rabbits, and 500 µL blood was collected through the ear vein. Sampling continued until 168 h at each indicated time point of $t$ = 0, 4, 8, 12, 24, 36, 48, 72, 96, 120, 144, and 168 h. The samples were kept at -80 °C until liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis. To assess relative bioavailability, Aricept® (10 mg/head; suspension in carboxymethylcellulose) was administered orally and 500 µL blood was collected from the ear vein. Sampling continued until 48 h at each indicated time point of $t$ = 0, 0.5, 1, 2, 4, 8, 12, 24, and 48 h. Samples were kept at -80 °C until LC-MS/MS analysis. The AChE activity was measured using a colorimetric ELISA kit from Abcam (Cambridge, MA, USA) according to the manufacturer's instructions. In this study, relative bioavailability (BA) was calculated from the following equation: relative BA = [$AUC_{transdermal}$/$AUC_{po}$ x $Dose_{po}$/$Dose_{transdermal}$]. The AChE activity at $t$ = 0 was defined as 100% basal activity and the AChE activity after donepezil treatment was measured to calculate AChE inhibition % versus basal activity.

### *4.7. Topical skin irritation in minipig*

The animals used in the present study included two male minipigs with an average weight of 20 kg obtained from Medi Kinetics (Pyeongtaek, Gyeonggi, Korea). Donepezil patch or placebo patch was assessed for potential toxicity and skin irritation in the minipig, with 7 days of single topical dosing of the agent. Four sites (12.5 mg/6.25 cm$^2$ per site) along the back of each animal were treated with a placebo patch and donepezil patch. Donepezil patch or placebo was applied to each site and covered with Tegaderm™ film (3M; Neuss, NRW, Germany) placed directly over the site. It was subsequently held in place with gentle wrapping of the minipig with Tensoplast® elastic tape (BSN Medical; Hamburg, Germany). Patch dressing status was checked daily during the trial. Seven days after application, the site was scored for irritation/redness and swelling/edema. Digital images and clinical signs were obtained and recorded at days 0, 1, and 3, after removal of the wrapping. Following the exposure, dermal irritation was evaluated using the method of Draize et al. (Prinsen et al. 2017). The classification of irritancy was obtained by adding the average erythema and edema scores for 24 and 72 h scoring intervals divided by the number of evaluation intervals (Sreejayan et al. 2010).

### *4.8. Analysis of plasma donepezil levels*

Accuracy and precision assessments of LC-MS/MS were performed at the lower limit of quantitation (LLOQ), lower concentration for quality control (LQC), medium concentration for quality control (MQC), and high concentration for quality control (HQC) of donepezil obtained from five repeated intra-day and inter-day observations. As the results of intra- and inter-day validation confirmed that accuracy (relative standard error [RSE]%) and precision (coefficient of variation [CV]%) were all within 15%, the accuracy and precision of the method were verified (Table 6).

Plasma donepezil concentrations were determined by LC-MS/MS with a slight modification of a previously reported method (Geerts et al. 2005; Bhateria et al. 2015). Briefly, donepezil HCl (10 mg in free form) was accurately weighed into a 20 mL volumetric vial and dissolved in methanol to prepare a working stock solution of 1000 µg/mL. An aliquot (100 µL) of working stock solution (1000 µg/mL) was transferred to a 1-mL E-tube and serially diluted with methanol to get working solutions ranging from 500 to 3.9 ng/mL. Depending on the calibration range and matrix (plasma) used, samples were prepared in the following manner. A suitable aliquot of samples or control matrix was accurately pipetted into a 2-mL tube and spiked with combined internal standard solution (amantadine 250 ng/mL; 300 µL). For calibration and quality control (QC) samples, a suitable volume of calibration and QC spiking solution was added. Standards were diluted to a final concentration ranging from 100 to 0.39 ng/mL. All samples were analyzed by LC-MS/MS using the HPLC system comprising Agilent Technologies 1200 series coupled to a 6430 Triple Quad LC/MS (Santa Clara, California, USA). Chromatography was performed on a Union UK-C18 3µ column (50 × 2.0 mm; Portland, Oregon, USA). A sample aliquot of 5 µL was injected onto the LC-MS/MS system with an auto-sampler followed by a needle wash using methanol. A gradient HPLC system (mobile ratio [A:B] = 25:75) was used with mobile phases (A) formic acid (0.05%), and (B) methanol (100%) at a total flow rate of 0.2 mL/min.

Data were obtained using proprietary software from the instrument manufacturer; peak area and quantitative data were generated by MassHunter Workstation Software Quantitative Analysis version B 04.00 (Agilent Technologies; Santa Clara, California, USA).

**Table 6: Accuracy (RSE%) and precision (CV%) of donepezil**

| Nominal conc. (ng/mL) | Intra-day validation | | | Inter-day validation | | |
|---|---|---|---|---|---|---|
| | Calculated conc. (ng/mL) | CV (%) | RSE (%) | Calculated conc. (ng/mL) | CV (%) | RSE (%) |
| 2 ng/mL | 2.03 | 9.35 | 1.68 | 1.99 | 13.74 | –0.60 |
| 5 ng/mL | 4.65 | 7.78 | –7.33 | 4.49 | 8.05 | –10.18 |
| 50 ng/mL | 48.68 | 3.70 | –2.64 | 45.41 | 7.56 | –9.18 |
| 100 ng/mL | 100.51 | 3.44 | 0.51 | 97.26 | 5.05 | –2.74 |

Accuracy and precision assessments of LC-MS/MS were performed at LLOQ, LQC, MQC, and HQC of donepezil obtained from 5 repeated intra-day and inter-day. As the result of intra- and inter-day validation confirmed that accuracy (RSE %) and precision (CV %) were all within 15%, the accuracy and precision of the method were verified

Abbreviations: RSE, Relative standard error; CV, coefficient of variation; LLOQ, lower limit of quantitation; LQC, lower concentration for quality control; MQC, medium concentration for quality control; HQC, high concentration for quality control.

### *4.9. Statistical analyses*

All data are expressed as mean±standard deviation (SD). All statistical analyses were performed using SigmaStat® 2.0 (SPSS, Chicago, Illinois, USA). Comparisons between the two groups were performed using Student's $t$-test. For comparisons of data from more than three groups at the same time point, one-way analysis of variance (ANOVA) was used. When ANOVA indicated a significant difference, differences were further evaluated using the Student–Newman–Keuls multiple comparison test. Correlation between two parameters was analyzed with Pearson's correlation. PK parameters were calculated using the bioavailability analysis program (BA Calc 2007). A $p$-value < 0.05 was considered statistically significant.

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