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Pharmacokinetics of Double-Layer Patches With Microneedles Containing Procyclidine and Rivastigmine in Guinea Pigs

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ABSTRACT

Reversible cholinesterase inhibitors have been investigated to address prophylactic and therapeutic outcomes against organophosphorus nerve poisoning, and procyclidine is concomitantly used to compensate for their adverse effects. We designed double-layer patches with microneedles composed of a cream layer for immediate release and a hyaluronic acid-based thin solid layer for sustained release. Rivastigmine and procyclidine were added to the two layers with different doses, and their transdermal absorption was explored in guinea pigs for the first time. The contents of hyaluronic acid and the number of microneedles were also examined to determine the optimal patches, which would achieve the mean target plasma concentrations of the two drugs for 72 h. Plasma concentrations of rivastigmine and procyclidine were determined using a validated HPLC–MS/MS method, and acetylcholinesterase (AChE) activity was monitored and correlated with plasma rivastigmine concentrations. The systemic exposure of both drugs was successfully characterised in guinea pigs, and the change was relevant in terms of different patches with microneedles. The inhibitory effect on AChE was prolonged despite the decay of rivastigmine concentrations. Finally, the preparation of a double-layer patch with microneedles was suggested, and the present results would help explain the prophylactic and therapeutic effects of rivastigmine and procyclidine against biochemical weapons.

1 | Introduction

A prophylactic treatment against cholinergic neurotoxic emergencies induced by organophosphorus toxins, such as soman and sarin, has been extensively investigated using sustained-release preparations containing procyclidine, an *N*-methyl-D-aspartate receptor antagonist possessing anticholinergic action and physostigmine, a reversible cholinesterase inhibitor [1–4].

Organophosphorus compounds bind to the hydroxy group of the active site of acetylcholinesterase (AChE), inhibiting the enzyme's activity irreversibly and resulting in parasympathetic

toxicity, such as mydriasis, tachycardia, salivation, seizures and severe brain damage [5]. The potential of the transdermal drug delivery system in this context holds promise for more effective and efficient treatments in emergencies, eliminating the need for medical assistance.

Pretreatment of reversible cholinesterase inhibitors such as physostigmine and rivastigmine can prevent the irreversible occupation of the enzyme by biological weapons and minimise neurological injury and behavioural adverse reactions. In addition, the drugs relieve anticholinergic toxicity in cases of procyclidine overdose [6]. Physostigmine has a short half-life of

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Summary

For both prophylactic and therapeutic purposes against neurotoxic agents such as sarin and soman, anticholinergic drugs are essential to preserve functional acetylcholinesterase reserves. Due to the short half-lives of the drugs, transdermal delivery systems offer a practical solution for sustained drug administration. Transdermal patches provide the additional advantage of being easily removed to discontinue drug exposure if necessary. To address both immediate and prolonged absorption, a bi-layer transdermal patch incorporating microneedles was developed and optimised for the co-delivery of rivastigmine and procyclidine. This design enables both rapid and sustained percutaneous absorption, aiming to maintain target plasma concentrations in guinea pigs.

20–30 min, an extensive clearance of 90–170 L/h and an absolute oral bioavailability of 11%–37% in humans [7]. By contrast, procyclidine exhibits a relatively long terminal half-life of 12 h, a total clearance of 4 L/h and a good bioavailability of 75% [8]. Meanwhile, rivastigmine, a new-generation acetylcholine esterase inhibitor compared to physostigmine, shows a longer half-life (1.5–2 h) and similar clearance (120 L/h) and oral absorption (35%) [9]. Rivastigmine exhibits a noncompetitive, slowly reversible inhibitory action on acetylcholinesterase and butyrylcholinesterase, with durations of 8.5 and 3.5 h for the former and the latter, respectively, via a pseudo-irreversible mechanism. It is known to have equivalent selectivity for both esterases [10]. In a study with acetylcholinesterase knockout mice, rivastigmine enhances the extracellular acetylcholine levels by inhibiting butyrylcholinesterase, which may explain the therapeutic significance of the drug in late-stage Alzheimer's disease. That is because acetylcholinesterase activity is prominently decreased, but butyrylcholinesterase action is preserved in the disease state [11].

Given the short half-lives of the acetylcholinesterase inhibitors, continuous drug administration is not just important but crucial. A prolonged administration could be achieved through subcutaneous osmotic pumps [4] and transdermal patches [1–3]. This strategy is of utmost importance for maintaining effective plasma concentrations, ensuring the reserve action on the esterases when exposed to organophosphorus toxins. Osmotic minipumps containing various doses of physostigmine and procyclidine were subcutaneously implanted in the backside of rats, and 0.072 mg/kg/h of physostigmine and 0.432 mg/kg/h of procyclidine were suggested to fulfill the protective effects for 3 days against subcutaneous soman intoxication [4]. Meanwhile, Myhrer et al. [12] reported moderate prophylactic injection doses of physostigmine (0.1 mg/kg) and procyclidine (3 mg/kg) for the cognitive side effects in soman-induced seizure rats.

Patches have been explored in various species, including guinea pigs [13], dogs [3] and monkeys [1, 2]. Song applied a patch-sized $4 \times 5 \text{ cm}^2$ containing physostigmine (3.6 mg) and procyclidine (14.2 mg) to guinea pigs [13]. A matrix-type patch with 1.5% physostigmine and 6% procyclidine was used in dogs [3]. Nam et al. [2] prepared a transdermal patch (25 cm^2) containing physostigmine (4.4 mg) and procyclidine (17.8 mg) and applied it

to rhesus monkeys (~4 kg body weight) challenged by VX nerve agent. Following a physiologically based pharmacokinetic and pharmacodynamic data analysis, they predicted the pharmacokinetics of both drugs and corresponding acetylcholinesterase inhibitory action in humans. Rhesus monkeys were also used by Cho et al. [1]. Various sizes of matrix-type patches, including those containing procyclidine (2.8–34.8 mg) and physostigmine (0.7–8.7 mg), were examined to determine the minimum effective drug concentrations that exert protective efficacy and survivability against soman intoxication.

Guinea pigs have been widely used to investigate the prophylactic effect of physostigmine combined with hyoscine [14], scopolamine [15] and the side effects of the cholinesterase inhibitor [16]. This extensive research, which provides a comprehensive understanding of the potential prophylactic effects of physostigmine in combination with other substances, is important. Recently, the Agency for Defense Development in Korea reported the antidotal and neuroprotective actions of a prophylactic patch against sarin and soman poisonings in guinea pigs [13].

Interestingly, only two publications related to rivastigmine, a drug with a longer half-life than physostigmine, against nerve gases have been searched. Intramuscular rivastigmine afforded a reasonable 24-h survival rate for guinea pigs challenged with soman [17]. Kassa and Karasova [18] reported that 1.2 mg/kg of the drug increased the LD_{50} value of soman by 12% and 42% ($p < 0.05$) in the absence and presence of memantine (20 mg/kg), respectively, in mice. In addition, oral and transdermal administrations of rivastigmine have been suggested as an alternative and adjunct due to physostigmine shortage for antimuscarinic intoxication by concomitant drugs without remarkable risk in clinics [19, 20].

This study investigated the transdermal absorption of both rivastigmine and procyclidine in guinea pigs. Although many patches have been used at varying doses adjusted according to patch size in previous literature, we introduce a double-layer patch with microneedles. With its unique design, which enables immediate and sustained penetration of drugs into the skin, this patch holds excellent promise for effective drug delivery. The number of microneedles and the amount of the two drugs in both layers were examined to determine their systemic exposure and maintenance. The primary outcome was to achieve plasma concentrations of rivastigmine and procyclidine of at least 1.3 ng/mL and 32 ng/mL, respectively, for 72 h, as reported in a previous study [13]. An inhibitory effect of rivastigmine on AChE was also examined, which was correlated with its plasma concentrations. The present results offer a hopeful prospect for future research and application to prophylaxis against a cholinergic nerve gas.

2 | Methods

2.1 | Reagents

Procyclidine and rivastigmine were obtained from Sigma-Aldrich (Seoul, Korea). Hyaluronic acid was purchased from Wuxi AppTech (Shanghai, China), and glycerin, stearic acid, cetyl alcohol, formic acid, diclofenac and tegocare were

commercially available from Bioclone (Seoul, Korea). Other agents, such as methanol and acetonitrile, were the highest grade from B&J Archives (Seoul, Korea). A colorimetric assay kit was purchased from Abcam (Cambridge, UK) for the measurement of AChE activity.

2.2 | Preparation of Double-Layer Patches With Microneedles

Figure 1 depicts schematic diagrams of the microneedle patches with a hyaluronic acid solid layer before (top left) and after (top right) applying cream, along with their corresponding actual final samples (middle).

The cream for the immediate-release layer was prepared in water and oil phases with the following compositions. For the aqueous phase, procyclidine and rivastigmine were added to 3 g of purified water and stirred. Then, 500 mg of glycerin and 150 mg of hyaluronic acid were added. For the oil phase, 450 mg of stearic acid, 400 mg of cetyl alcohol and 150 mg of tegocare were added to 350 mg of mineral oil. The oil and water phases were heated to 70°C while stirring, and then the water phase was added to the oil phase. After stirring for another 10 min, the mixture was left at room temperature to set and form a cream. The prepared cream was evenly applied to the previously prepared patch and then to the guinea pig's skin.

The sustained-release layer was formulated by dissolving hyaluronic acid in deionised water to produce a 2% hyaluronic acid solution. Procyclidine (7.15 mg) and rivastigmine (1.80 mg) were added to 0.5 mL of the 2% hyaluronic acid solution, and the resulting mixture was applied to each microneedle patch unit.

A replica mould of the microneedles from a commercial derma stamp (Larcobaleno Co. Ltd., Seoul, South Korea) was used for skin insertion. Each microneedle measures 1000 µm in height, with a base width of 2000 µm and a tip radius of 10 µm—a flexible substrate served as the backing layer for the microneedle patches. To create the backing layer, polydimethylsiloxane (PDMS, Sylgard 184; Dow Corning, Midland, MI, USA) was prepared by mixing the elastomer and curing agent at a 10:1 ratio. This mixture was poured onto a master mould with a 2.5×1.5 cm² rectangular area and cured at 60°C for 2 h. After curing, the PDMS backing layer was separated from the master mould. Then, individual microneedles were inserted into the designated positions on this backing substrate, maintaining flexibility (Figure 1).

2.3 | Optimisation of Double-Layer Patches With Microneedles

In the first trial, different amounts of rivastigmine and procyclidine were added to the thin films and creams to examine the systemic exposure of the two drugs (Table 1). Microneedles (117 in a 2.5×1.5 cm² rectangle) were embedded in the PDMS backing layer.

Second, the effect of hyaluronic acid on the transdermal adsorption of rivastigmine and procyclidine was evaluated by adjusting its amounts to 1% and 2% in the cream layer without microneedles. In this trial, the impact of microneedles was also examined.

In the third attempt, the absorption pattern regarding the number of microneedles was tested. Instead of 117 microneedles in a 2.5×1.5 cm² rectangle used in previous attempts, a patch

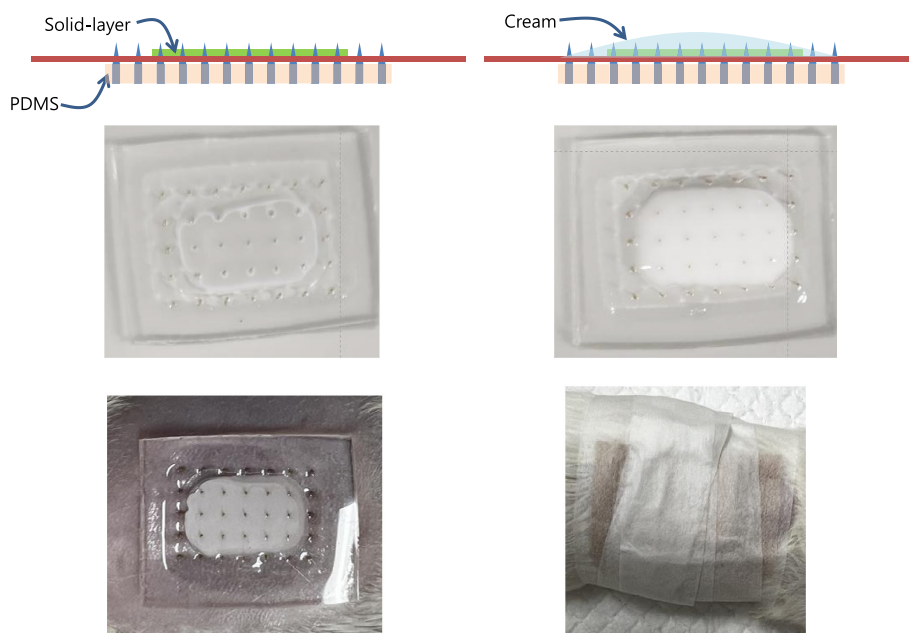


FIGURE 1 | A schematic diagram of double-layer patches with microneedles (top) and actual samples in the absence (middle left) and presence (middle right) of the cream layer. The bottom left shows a photo in which the preparation is applied to the dorsal skin of guinea pigs, and the bottom right illustrates the final state of the animal wrapped with an adhesive bandage.

TABLE 1 | Compositions of rivastigmine and procyclidine in a cream (immediate-release layer) and a solid thin layer containing hyaluronic acid (sustained-release layer).

Preparation	Rivastigmine (mg)		Procyclidine (mg)	
	Cream	Solid thin layer	Cream	Solid thin layer
Patch 1	1.8	1.8	7.15	7.15
Patch 2	3.6	1.8	14.3	7.15
Patch 3	3.6	3.6	14.3	14.3

containing 35 microneedles in the same area was prepared by reducing the resolution to approximately one-quarter. All attempts focused on maintaining the mean plasma concentrations described in the Introduction above.

2.4 | Pharmacokinetic Study in Guinea Pigs

In total, 36 male guinea pigs, including 20% excess animals, were obtained from Hana Bio (Seoul, Korea). The animals were 5 weeks old (300–340 g) on arrival and were allocated to six sets of experiments as described in Section 2.3, according to their body weight after 1 week of acclimation. The animals were housed at a medium-sized animal facility at Chung-Ang University. The room's temperature and humidity were maintained at $23 \pm 3^\circ\text{C}$ and $50\% \pm 10\%$, respectively. The light, with an illumination level of 300 Lux, was turned off for 12 h.

They were anaesthetised with a peritoneal injection of alfaxalone (25 mg/kg) and dexmedetomidine (0.1 mg/kg), and the hair on the backside was removed. The transdermal patch mentioned above was attached while the skin was stretched tightly, and an adhesive bandage was applied to the patch. A heparinised blood sample (0.2 mL) was collected from the subclavian vein at 0, 2, 18, 24, 42, 48 and 72 h, and the plasma was stored in a freezer until analysis. Guinea pigs were euthanised with CO_2 gas after the experiments.

An analytical method using HPLC–MS/MS determined plasma concentrations of procyclidine and rivastigmine. Briefly, 270 μL of acetonitrile, including an internal standard, was added to 30 μL of plasma. After vortexing for 10 s, plasma proteins were removed by centrifugation at 15,000 rpm for 10 min, and 3 μL of the supernatant was injected into LC–MS/MS (API 5000, AB Sciex, Toronto, Canada). An Agilent column, InfinityLab Poroshell 120 EC-C18 (3.0 $\times$ 150 mm, 2.7 μm), was used as the stationary phase and maintained at 40°C . Distilled water and acetonitrile (7:3) containing 0.1% formic acid were used as the mobile phase, with a flow rate of 0.2 mL/min. Calibration graphs were derived from the ratios between the areas under the analyte peaks and the IS peak using linear regression. To ensure analytical objectivity and minimise errors during the analysis period, the plasma samples were randomly selected for analysis. The results were interpreted by a colleague who was not involved in the sample processing.

2.5 | Analytical Method Validation and Stability of Rivastigmine and Procyclidine in Plasma

Analytical method validation was performed for determining rivastigmine and procyclidine in guinea pig plasma. Calibrations ranged from 1 ng/mL to 500 ng/mL for each drug in plasma. Extraction recovery was checked to ensure a sufficient clean-up procedure. Intra- and interday assay accuracy and precision were assessed using a calibration curve to estimate the concentrations of the quality controls (lower limit of quantification, 1 ng/mL; low, 4 ng/mL; intermediate, 70 ng/mL; and high, 400 ng/mL). The accuracy (%) was determined by comparing the measured concentration, obtained using the calibration curves, with the nominal concentration of the two drugs added to the blank plasma. Precision was expressed as the coefficient of variation (CV, %), calculated by dividing the standard deviation by the mean accuracy. Acceptable criteria were within 15% of precision and accuracy, except the LLOQ, which was within 20% [21].

The stability of the plasma samples was tested under different conditions. Control samples for rivastigmine and procyclidine were prepared at concentrations of 10 and 200 ng/mL. Room temperature and long-term stability were examined after 4 h of storage at room temperature and 3 weeks at -70°C , respectively. The stability of three freeze–thaw cycles was assessed after being transferred from -70°C to room temperature. The stability of the analytes in extracts was also evaluated after 24 h of storage at 10°C . The peak areas of the analyte under various conditions were compared with those of a freshly prepared plasma sample, and the acceptable range for stability was determined to be $\pm 15\%$ [21].

2.6 | Stability of Rivastigmine and Procyclidine in Patches

For the 6-month stability of the two drugs in the patch, a total of nine sets of cream and the solid layer were individually prepared and stored at room temperature. The container for the cream was tightly sealed to prevent drying. The contents of rivastigmine and procyclidine in the three sets were examined in the cream and the solid layer at days 0, 90 and 180, respectively. About 100 mg of cream and the entire solid layer were separately dissolved in a mixture of distilled water and acetonitrile (1:1) each day, and diluted with the same mixture to obtain 100 ng/mL of each drug. Calibrations ranged from 10 to 500 ng/mL for each drug in the mixture. The contents of the two drugs were measured to ensure their stability in the preparations.

2.7 | Acetylcholinesterase Activity

Acetylcholinesterase activity in whole blood was determined as described previously using a colourimetric assay kit (Abcam, Cambridge, UK) [22]. Whole blood was collected and frozen until analyses. Blood cells were lysed by repeated freeze–thaw cycles, and the lysates were diluted 40-fold in 50 mM sodium phosphate buffer (pH 8.0). For each reaction, 50 μL of diluted whole blood lysate or acetylcholinesterase standards (0–1000 mU/mL) was added to a 96-well plate, followed by 50 μL of reaction mixture containing 50 mM sodium phosphate (pH 8.0), 5,5'-dithio-bis-(2-nitrobenzoic acid) and acetylthiocholine,

resulting in a final volume of 100 μ L per well. Plates were incubated at room temperature for 30 min in the dark, and absorbance was recorded at 410 nm using a microplate reader (Epoch, BioTek, Winooski, VT, USA). A total of 20 μ M of ethopropazine was added to each well to inhibit butyrylcholine esterase. Blank wells containing assay buffer alone were included, and their absorbance values were subtracted from those of samples and standards. Absorbance values of the samples were converted to enzyme activity using a standard curve.

2.8 | Statistics

The pharmacokinetic parameters obtained from each experiment were statistically compared using an analysis of variance (ANOVA) for three groups and a Student's *t*-test for two groups. Statistical significance was assessed at a level of $p < 0.05$. The normality test and the homogeneity of variance of each group were initially checked to ensure the appropriateness of the statistical test.

2.9 | Ethics Issues

The Institutional Animal Care and Use Committee approved the study at Chung-Ang University (approved No. 202301020053). The animals were cared for by the principles outlined in the National Institutes of Health Guide for the Care and Use of

Laboratory Animals. The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy (BCPT 2023 policy) for experimental and clinical studies [23].

3 | Results

3.1 | Systemic Exposure of Rivastigmine and Procyclidine

The bottom left of Figure 1 shows a photo taken immediately after applying the double-layer patch with microneedles to a guinea pig's skin, and the bottom right shows the animal wrapped in an adhesive bandage in its final state.

The patches were composed of various combinations to explore the systemic exposure of rivastigmine and procyclidine, corresponding to their contents in the cream and solid thin layers, as shown in Table 1. Figure 2 depicts the time courses of rivastigmine and procyclidine concentrations following a single transdermal application of patches containing both drugs.

When a total amount of 3.60, 5.40 and 7.15 mg of rivastigmine was applied transdermally to guinea pigs, rapid initial absorption was observed 2 h after administration, which was attributed to the drug in the cream layer. The plasma concentrations reached peaks of 12 ± 4 , 50 ± 12 and 73 ± 20 ng/mL at 18 h, respectively (Figure 2A). AUC_{72h} , representing systemic exposure,

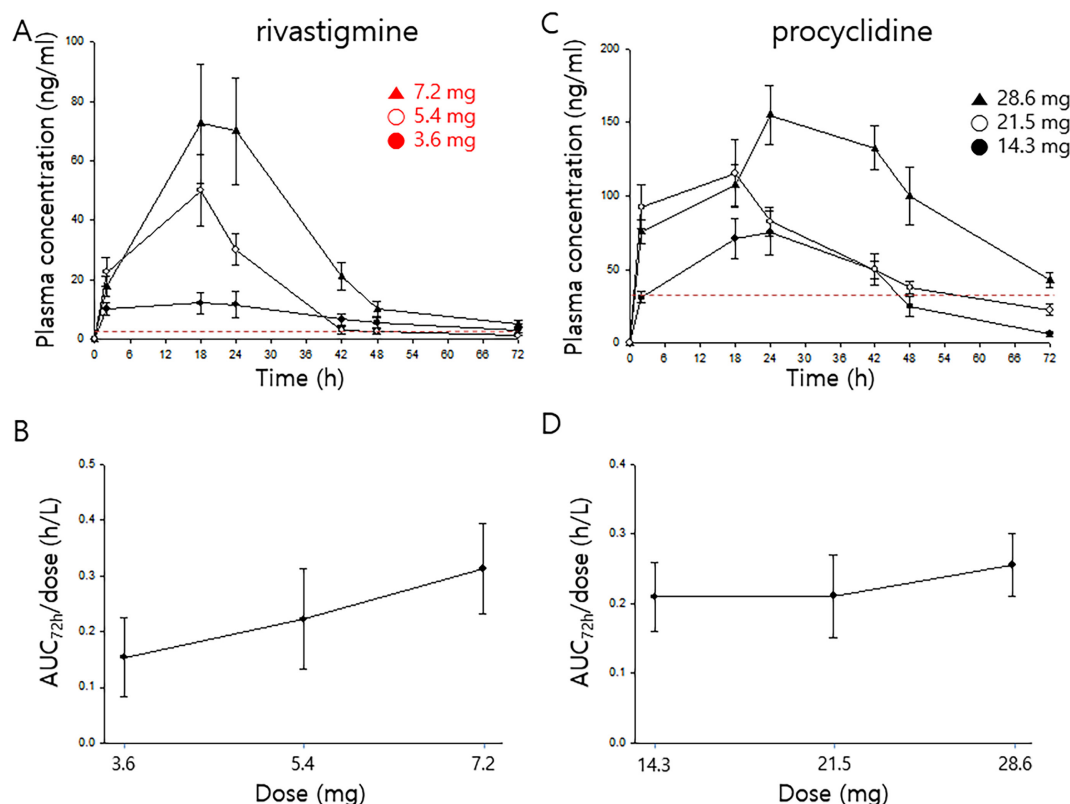


FIGURE 2 | Mean time courses of plasma rivastigmine (A) and procyclidine (B) concentrations following a single transdermal administration of double-layer patches with microneedles in guinea pigs. The compositions of the two drugs in the cream and the solid layers are listed in Table 1. The number of microneedles embedded in flexible PDMS was 117 (13×9) in 4.7 cm^2 ($2.6 \times 1.8 \text{ cm}$). The dotted lines indicate the target minimum concentrations. The bottom lines illustrate the total doses vs. the dose-normalised systemic exposure (AUC_{72h}) of the two drugs (C and D). Mean body weights of low-, middle- and high-dose groups are 353 ± 21 g, 345 ± 18 g and 356 ± 25 g, respectively. Data are mean $\pm$ SD ($n = 5$).

was 154 ± 70 , 1198 ± 486 and 2257 ± 577 ng·h/mL, respectively. Figure 2B illustrates the dose-normalised systemic exposure as a function of dose. As the dose increased, the systemic exposure standardised to its corresponding dose tended to increase; however, there was no statistical significance. Therefore, there is no significant difference in rivastigmine absorption in the current rivastigmine dosing range.

As shown in Figure 2C, procyclidine also showed a similar profile to rivastigmine in transdermal absorption, and the AUC_{72h} was 3007 ± 716 , 4520 ± 1285 and 7322 ± 1287 ng·h/mL when administered at 14.30, 21.45 and 28.60 mg, respectively. Systemic exposure proportionally increased with increasing the dose, and no significant difference was found in absorption in the present dosing range of procyclidine (Figure 2D).

3.2 | Impact of Hyaluronic Acid in the Cream Layer Without Microneedles

When applying a patch without a microneedle, the peak concentration was observed 2 h after topical administration, as shown in Figure 3, and then rapidly decreased in the systemic circulation. There was no effect of hyaluronic acid contents on the absorption of both drugs. In addition, the maintenance of plasma concentration through continuous absorption, as observed when microneedles were used together, was not evident, and AUC_{72h} was only about 6.5% for rivastigmine and about 10.8% for procyclidine compared to the patch using microneedles.

3.3 | Role of the Number of Microneedles

Figure 4 represents the time course of plasma rivastigmine and procyclidine concentrations following administration of a double-layer patch with reduced microneedles, 35 needles embedded in a 2.5×1.5 cm² rectangular area, which was conducted

to evaluate the number of microneedles in the topical absorption of the drugs. The plasma concentrations over time were downshifted compared to the patches containing 117 microneedles, and the AUC_{72h} was approximately 42.9% and 45.8% for rivastigmine and procyclidine, respectively. However, a quarter of the microneedles remained sufficient to maintain the mean target concentrations for 72 h.

Rivastigmine showed rapid absorption, reaching a maximum plasma concentration of 11 ± 1 ng/mL at 18 h after skin application. After maintaining the plateau for up to 42 h, it disappeared with a half-life of 33 ± 12 h and reached approximately 4 ± 1 ng/mL at 72 h. Procyclidine reached the first peak plasma concentration of 43 ± 13 ng/mL at 43 h. It was maintained without a statistically significant difference until 48 h, after which it disappeared with a half-life of 25 ± 5 h. The plasma concentration at 72 h was 24 ± 6 ng/mL. The plasma concentrations over time represented a typical pattern of transdermal preparations. One may speculate that applying reduced needles would provide a beneficial consequence, although there has been no objective assessment of skin irritation and pain.

3.4 | Method Validation and Stability of Rivastigmine and Procyclidine in Plasma

The calibration curves for quantifying rivastigmine and procyclidine in plasma demonstrated good linearity ($r^2 > 0.998$) in the 1–500 ng/mL range. The extraction recovery from plasma was 88% and 90% for the former and the latter, respectively, indicating that the extraction method was suitable. The intra- and interday precisions of rivastigmine QC samples were $<8.5\%$ and 9.4% , and the accuracies were 95.2%–103.4% and 90.5%–107.4%, respectively. The intra- and interday precisions of procyclidine QC samples were $<10.3\%$ and 11.2% , and the accuracies were 92.3%–95.6% and 90.4%–102.5%, respectively. Therefore, the analytical method used in this study was

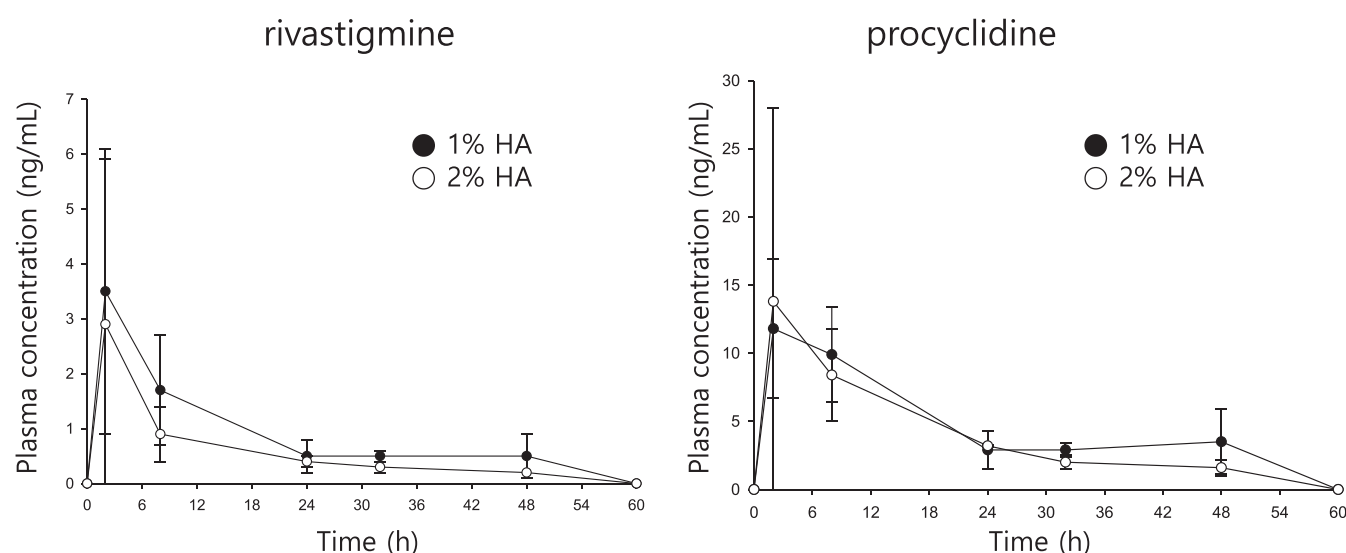


FIGURE 3 | Time courses of plasma rivastigmine and procyclidine concentrations following a single transdermal application of double-layer patches without microneedles in guinea pigs. The cream layer included 1% (closed circle) or 2% (open circle) of hyaluronic acid. Mean body weights of 1% and 2% hyaluronic acid groups are 346 ± 25 g and 360 ± 26 g, respectively. Data are mean $\pm$ SD ($n = 5$).

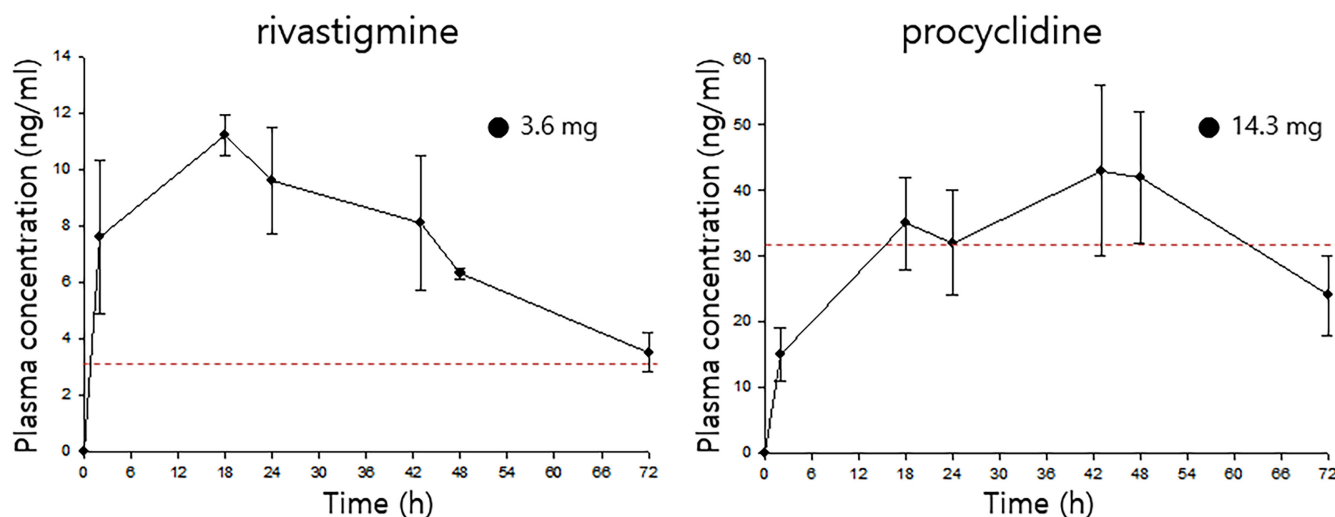


FIGURE 4 | Time courses of plasma rivastigmine and procyclidine concentrations following a single transdermal application of double-layer patches with microneedles with low (35: 7×5, open circle) density in 4.7 cm² (2.6×1.8 cm) in guinea pigs. The dotted lines indicate the target minimum concentrations. Mean body weights are 359 ± 26 g. Data are mean ± SD (*n* = 5).

TABLE 2 | Contents of rivastigmine and procyclidine in cream and the solid layer (mean ± SD, *n* = 3).

	Test drug	Day 0	Day 90	Day 180
Cream	Rivastigmine	102 ± 4	107 ± 6	110 ± 1.8
	Procyclidine	98 ± 6	103 ± 7	108 ± 2.3
Solid layer	Rivastigmine	98 ± 3	99 ± 6	104 ± 11.6
	Procyclidine	95 ± 5	96 ± 7	98 ± 8.6

suitable for quantifying both drugs in plasma. In addition, after spiking the two drugs into plasma, room temperature stability for 4 h, three-cycle freeze–thaw stability, 24 h for posttreatment stability and 4-week frozen stability were all within 15%, indicating that both drugs remained stable during storage and analysis.

3.5 | Stability of Rivastigmine and Procyclidine in the Patch

Six-month stability results showed that both drugs remained stable within the formulation (Table 2). The content in the cream layer tended to increase slightly over time; however, no statistical difference was observed.

3.6 | Acetylcholinesterase Activity

As shown in Figure 5A, AChE activity continued to decrease following patch administration, reaching 72% ± 21% at 24 h and 61% ± 21% at 72 h. The correlation between the inhibitory effect of AChE activity and the plasma concentration of rivastigmine exhibited hysteresis (Figure 5B), that is, although the concentration decreased after reaching its maximum, the inhibitory effect persisted, delaying recovery.

4 | Discussion

The PDMS-based microneedle patch is flexible, providing excellent skin adhesion when applied to guinea pig skin (Figure 1, bottom left). The patch was fixed to the guinea pig's dorsal skin for 72 h, and the marks on the skin caused by the microneedles were evident even after the patch was removed. However, the microneedle did not cause bleeding or any adverse reactions, such as skin inflammation. Even the lowest dose in Table 1, which contained 3.6 mg of rivastigmine and 14.3 mg of procyclidine in the double-layer patches with microneedles, was sufficient to maintain the mean plasma concentrations of the former and the latter drugs, 1.3 and 32 ng/mL, for 72 h after topical administration in guinea pigs [13].

Because rivastigmine and procyclidine have high water-solubility, a cream containing more than 70% water was selected. The cream layer was expected to cause an initial rapid increase in plasma concentrations of the two drugs through fast absorption. Meanwhile, the solid layer was designed to slowly and continuously release the drugs from the hyaluronic acid-based layer using the moisture of the cream. Hyaluronic acid, a natural linear glycosaminoglycan consisting of *N*-acetyl-D-glucosamine and D-glucuronic acid with monosaccharide units [24], is found in the eyes and joints at the highest concentrations. It has been used as a visco-supplementary in eye surgery [25] and as a lubricant for the moving joints of humans [26]. The substance is also explored for sustained drug delivery of proteins due to its effective encapsulating and releasing properties of hydrophilic macromolecules, such as enzymes and hormones. Hyaluronic acid has also been a good solution for a sustained release of antibiotics with a short half-life [27]. In this sense, we successfully applied hyaluronic acid to a prolonged-released layer, maintaining adequate plasma levels of rivastigmine and procyclidine for 3 days.

At the beginning of this study, only a cream layer with the two drugs was tested on guinea pigs. However, their plasma

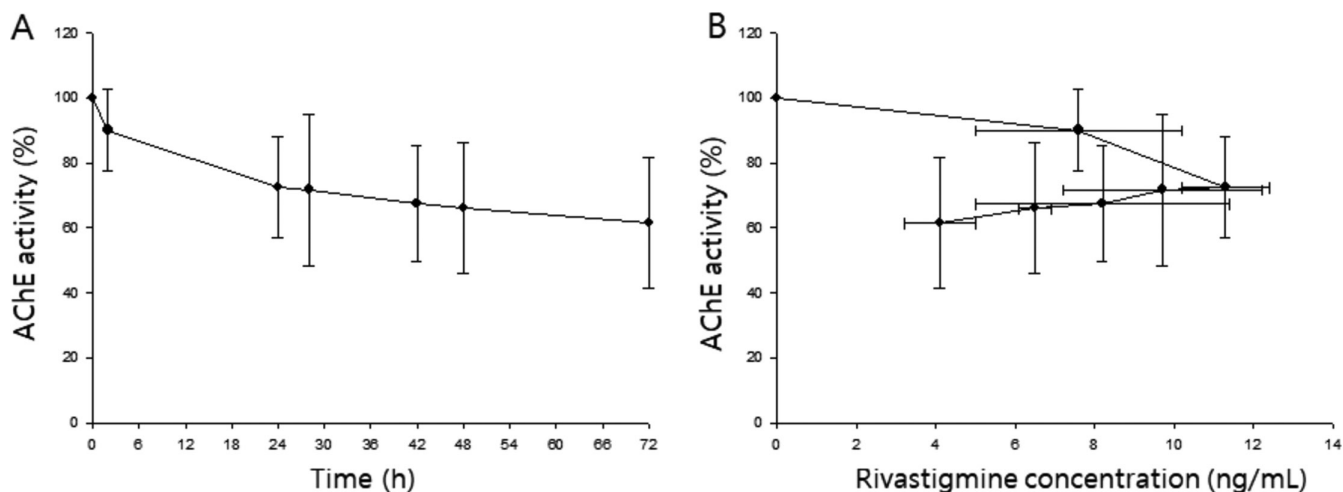


FIGURE 5 | (A) Time courses of acetylcholinesterase activity following a single transdermal application of double-layer patches with microneedles with low (35: 7×5) density in 4.7 cm² (2.6×1.8 cm) in guinea pigs. (B) Correlation between AChE activity vs. rivastigmine concentration. Data are mean ± SD (*n* = 5).

concentrations disappeared at approximately 10 h, following a slight absorption at 2 h after transdermal application. In addition, the solid layer did not work without the cream layer: systemic exposure to the drugs was merely found, which may be attributed to the fact that the guinea pig's skin does not have enough moisture to dissolve hyaluronic acid for releasing the drugs from the dried layer, unlike human skin (Figure S1). The hydrophilic cream layer is suspected of exerting dual effects: moisturising the solid layer and accelerating the initial transdermal absorption. It was confirmed that microneedles' physical assistance was required to absorb two drugs through guinea pig skin properly, and the contents of hyaluronic acid in the cream layer did not affect absorption without microneedles (Figure 3). Microneedles might also be needed to accelerate the transdermal delivery of the two drugs when used in humans. One may speculate that applying a reduced number of needles would provide a beneficial consequence, although there has been no objective assessment of skin irritation and pain. As shown in Figure 4, approximately one-third of microneedles were found to be effective for the mean plasma target concentrations of both drugs. On the one hand, their concentrations should be high enough to represent a prophylactic effect against a nerve poison. On the other hand, they must also be sufficiently low to minimise any side effects. Therefore, the microneedles' resolution was also optimised.

The thickness of the dermis and epidermis in guinea pigs is reported to be similar to that in humans, unlike in rabbits or rats [28]. It is known that, like other rodents, guinea pigs have almost no sweat glands, which explains why the skin absorption of the two drugs with hyaluronic acid is minimal. Scott and Corrigan [29] conducted in vitro tests on the percutaneous absorption of diquat, an herbicide with a quaternary amine structure and high water-solubility, using the skin of guinea pigs, mice, rats, rabbits and humans. Although absorption was approximately eight times faster in guinea pigs than in humans, lag times were similar. Both drugs used in this study are also highly hydrophilic salt forms. Therefore, caution should be taken to avoid overestimating the results in guinea pigs relative to those in humans, considering the results of the diquat study.

Although the primary focus of the present study was to explore the optimal dosage of the two drugs in the double-layer patches with microneedles containing rivastigmine and procyclidine, it would also be important to evaluate the protective effect of the patches against nerve toxins, including their inhibitory actions on acetylcholinesterase. After patch application, AChE activity was continuously reduced by 40% over 72 h. It did not recover despite decreased rivastigmine concentrations (Figure 5), which suggests that rivastigmine concentration does not directly correlate with its AChE inhibitory effect, but instead requires an effect compartment for its effect to manifest, representing hysteresis. The protective effect of the patch against cholinergic nerve gas toxicity may last longer, which should also be beneficial against the prolonged effects of an active metabolite of organophosphorus poisons. Further study is underway to determine the time for recovery of AChE activity after patch removal and for the reapplication of the patch.

Currently, there is no literature reporting directly measured binding kinetics of rivastigmine to AChE. The only available data are from computational modelling using molecular docking techniques [30]. The association (k_{on}) and dissociation (k_{off}) rate constants of rivastigmine on AChE were 1.67×10^9 M/s and 8.37×10^3 s, respectively, and the binding constant (K_d) was 5.1μ M. Further in-depth study on the binding kinetics in species, including guinea pigs, is required.

The only study related to the effects of rivastigmine after an organophosphorus poison challenge using guinea pigs has been published by Aracava et al. [17]. After a single subcutaneous injection of soman (42 μ g/kg), all animals exhibited cholinergic crisis symptoms, including miosis, clenching or grinding of teeth, hypersecretion and diarrhoea within 10 min, followed by tonic-clonic seizures and gasping in 5 min. All guinea pigs died within 24 h. However, over 80% of the animals survived at 8 mg/kg of rivastigmine for more than 72 h when the drug was administered intramuscularly at a range of 0.1–16 mg/kg at 30 min following soman challenge, and EC_{50} was ~ 0.73 mg/kg. The 12 mg/kg dose used in the present study was comparable to the previous dose by Aracava et al. [17].

Because no published data on the systemic exposure of rivastigmine and procyclidine after any route of administration in guinea pigs have been found, a pharmacokinetic study has been under investigation. The intramuscular pharmacokinetic results will be compared with current data, which may provide additional information on the therapeutic and prophylactic effects of the transdermal patches.

The relatively large variation in plasma concentrations might be due to the patch's varying degree of fixation depending on the guinea pig's movements over the 72-h period. The initial application of the patch was performed under anaesthesia and secured with a medical bandage. The bandage wrapped around the entire abdomen as shown in Figure 1. At that time, if the bandage was wrapped too tightly, it could make breathing difficult or interfere with normal gastrointestinal motility; therefore, the patch was adjusted to fit the circumstance of the abdomen when the animal was maximally inhaling. Movement after waking from anaesthesia is thought to have affected the patch's position and needle depth. When applied to humans, variation between individuals owing to voluntary movement is expected to be smaller than in animals. Although variation between patches may also be a factor, the drug content and stability in the two layers do not appear to vary significantly between formulations.

Rivastigmine is started at 1.5 mg bid for the treatment of Alzheimer's disease, increased to 3–4.5 mg bid every 2 weeks and then increased to 6 mg bid within 2 weeks depending on the patient's condition. For the treatment of Parkinson's disease dementia, the dosage remains the same and the increase period is 4 weeks [31]. The oral clearance in humans is approximately 1.8 L/min; therefore, when administered at 6 mg twice daily, the mean concentration can be calculated to be 4.63 µg/mL. According to previous reports [31], oral administration of 46 mg of rivastigmine causes vomiting, urinary incontinence, hypotension, impaired judgement and loss of consciousness, and 90 mg exerts respiratory distress [32]. Meanwhile, when procyclidine is used in patients with Parkinson's disease, the dosage should be adjusted according to the patient's age, response level and aetiology. When used alone, the starting dose is 2.5 mg tid, increasing to 5 mg three times a day. For extrapyramidal symptoms caused by sedatives used to treat psychiatric disorders, a daily dose of 10–20 mg is used. Side effects due to parasympathetic nervous system inhibition, such as tachycardia and urinary retention, should be carefully monitored, and close observation is also necessary for patients with hypotension [33].

If the patch developed in this study is to be applied to humans, a clinical trial should first be conducted to determine the transdermal bioavailability of the two drugs. Furthermore, based on the correlation between plasma exposure and AChE inhibition in humans, one can calculate the required dose for patch application. Considering the target duration, the amount of drug in each layer of the patch can be adjusted, and the composition of the two layers can also be optimised as needed. The use of a patch for prophylaxis prior to neurotoxin exposure will vary depending on the size of the patch to be applied. However, possible administration sites include the abdomen or forearm. Meanwhile, the patch could also be

applied to animals or humans exposed to organophosphorus agents, which irreversibly bind to AChE. However, the relatively slow absorption of the transdermal preparation has to be considered for its application.

5 | Conclusion

Double-layer patches with microneedles were prepared, including rivastigmine and procyclidine. They consisted of a cream layer and a thin solid layer, designed to provide immediate and sustained release, respectively. The patches were applied to the dorsal area in guinea pigs, and the plasma concentrations of the two drugs were monitored for the first time. The contents of rivastigmine and procyclidine were explored in various combinations in both layers, and microneedles were used to enhance the transdermal delivery of the drugs. The systemic exposure of both drugs was successfully determined after applying the patches, and a correlation was observed between acetylcholinesterase activity. It is impossible to directly challenge humans with neurotoxins to assess the efficacy of drugs that exhibit protective effects. Therefore, the correlation between the percutaneous pharmacokinetics of rivastigmine and its inhibitory effect on AChE activity, as revealed in this study using guinea pigs, holds significance. Suppose these results are combined with data on percutaneous absorption in humans and information on the difference in AChE activity between the two species. In that case, it will be possible to determine the doses of the two drugs expected to exert protective effects in humans. The utility of the patches is being investigated to explore the prophylactic effect against biochemical weapons.

Author Contributions

M.M. and J.W. conducted the animal study and drafted the manuscript. Y-W.L. and S.J. prepared the patches. Y.Y., J-H.J. and W.K. designed the study and revised the manuscript.

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Ethics Statement

The Institutional Animal Care and Use Committee approved the study at Chung-Ang University (Approved No. 202301020053). The animals were cared for according to the principles outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data will be made available on request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Time course of plasma rivastigmine and procyclidine concentrations following a single transdermal application of a solid-layer patch without the cream in guinea pigs. Data are $\pm$ SD ($n = 5$).