In vitro and in vivo evaluations of topically applied capsaicin and nonivamide from hydrogels

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Abstract

The purpose of this study was designed to investigate the in vitro and in vivo skin absorption of capsaicin and nonivamide from hydrogels. Various commercialized creams of capsaicin were also compared with hydrogels. Both skin stripping technique and Mexameter® were applied to evaluate the level of capsaicin and nonivamide retained in stratum corneum (SC) and skin erythema in vivo. The partition of drug between skin and the hydrogel matrix was considered to play an important role in the permeation process. The in vitro permeation of capsaicin from hydrogels depends on the physicochemical nature and the concentration of the polymer used. The incorporation of nonionic Pluronic F-127 polymer into hydrogels resulted in a retarded release of capsaicin. On the other hand, the in vitro capsaicin permeation showed higher levels in cationic chitosan and anionic carboxymethyl cellulose (CMC) hydrogels than cream bases. The permeation of nonivamide was retarded at the late stage of in vitro application. The inter-subject variation was more significant in the in vivo study than in vitro skin permeation experiments. The cream induced in vivo skin erythema depending on the drug concentration, however, the dose-dependence was not observed in hydrogels. Nonivamide-treated skin showed stronger erythema than capsaicin-treated skin. The present study indicates that there is a moderate correlation between in vitro skin permeation and in vivo erythema responses of topically applied capsaicin and nonivamide. The correlation between drug amount in SC and skin erythema test in vivo was also observed. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Capsaicin; Nonivamide; Topical application; Skin; Hydrogels

1. Introduction

Capsaicin (8-methyl N-vanillyl-6 nonenamide), the active compound of hot peppers of the genus Capsicum, exhibits broad bioactivity (Tominack and Spyker, 1987), including antinociception, antihypertension and lipid-lowering activities (Hayes
et al., 1981; Wang et al., 1984; Clozel et al., 1985). Capsaicin is also used topically to treat various diseases such as rheumatoid arthritis, osteoarthritis, diabetic neuropathy and postherpetic neuralgia (Fusco and Giacovazzo, 1997). Nonivamide (N-nonanoyl vanillylamide), so-called synthetic capsaicin, is a substitute of capsaicin which has similar chemical structure and pharmacological effects as those of capsaicin (Chen et al., 1992). Due to their high degree of first-pass metabolism (Donnerer et al., 1990), the half-life of capsaicin and nonivamide is very short (Fang et al., 1996). Topical application circumvents the hepatic metabolism and thus is suitable to develop delivery systems to attain both systemic and local effects for capsaicin and nonivamide.

It is well known that vehicles used in the topically applied formulations can greatly influence the rate and extent of drug permeation across the skin. The present study is to perform the in vitro permeation of capsaicin and nonivamide as well as to select the optimal formulations for in vivo study in humans. In our earlier in vitro permeation study (Fang et al., 1995), a higher extent of permeation for nonivamide was observed with Carbopol hydrogel than the other vehicles such as creams and ointments. Three hydrogels prepared by polymers, including Pluronic F-127, chitosan and carboxymethyl cellulose (CMC) which possess neutral, cationic and anionic charge, respectively, were employed to optimize the in vitro formulation of capsaicin and nonivamide. The in vitro permeation of several commercialized creams of capsaicin across skin were also measured to compare with those of hydrogels.

The present study was performed in compliance with two guidelines issued by Food and Drug Administration (FDA) recently to evaluate the in vitro and in vivo skin absorption of topically applied capsaicin. The guideline for in vitro topical application of semisolids discussed utilization of artificial membrane to assess the in vitro release of drug (Flynn et al., 1999). Guideline for in vivo application illustrated determination of dermato-pharmacokinetic characterization (Shah et al., 1998). The approach of dermato-pharmacokinetics requires measuring the concentration of drug in the skin, directly or indirectly related to the drug’s therapeutic action. The most promising dermatopharmacokinetic method involves assessment of drug concentrations in stratum corneum (SC) through skin stripping technique (Shah et al., 1998). It is commonly employed in the in vivo evaluation of capsaicin and nonivamide in humans because SC is the rate-limiting barrier for drug permeation into the skin. The concentration of drug in SC thus provide meaningful information for comparative evaluation of topical dosage forms.

Topical application of capsaicin and nonivamide causes marked vasodilation, resulting in the skin erythema on the treated skin area. The level of erythema elicited by capsaicin and nonivamide from various vehicles is, therefore, determined in the present in vivo study by Mexameter®, which is an erythema index meter based on the measurement of hemoglobin in the skin (Edwards, 1995). It enables the quick, accurate and reproducible determination of the skin color and condition. After a series of in vitro and in vivo evaluations, the kinetic and dynamic aspects of capsaicin and nonivamide were examined. These assessments may act as a model process for assessing the drug permeation and therapeutic activity of topical application.

2. Materials and methods

2.1. Materials

Capsaicin, chitosan, carboxymethyl cellulose sodium salt (CMC-Na) and CMC ammonium salt (CMC-NH₄) was purchased form Wako Chemical Co. (Japan). Nonivamide was supplied by Tokyo Kasei Co. (Japan). Pluronic F-127 was obtained from Sigma Chemical Co. (USA). Zostrix® creams with 0.025 or 0.075% capsaicin were purchased from Genderm Co. (USA). Zoscum® cream with 0.025% capsaicin was a gift from China Chemical and Pharmaceutical Co. (Taiwan). Capsaicin-C® cream with 0.075% capsaicin was a gift from Panbiotic Laboratories Co. (Taiwan). The cellulose membrane (Spectra-por® 2, molecular weight cut-off = 12,000–14,000) was supplied by Spectrum Co. (USA). All other chemicals and solvents were of analytical grade.
2.2. Preparation of hydrogels

To prepare Pluronic hydrogels, the required amount of polymer and drug were dispersed in pH 4 citrate-phosphate buffer with continuous stirring for 1 h. The partially dissolved Pluronic solution was stored in the refrigerator (5 °C) until all the polymer was completely dissolved (approximately 24 h). For the preparation of chitosan and CMC hydrogels, the calculated amount of polymer and drug were added into pH4 buffer with continuous stirring for 1 h. The hydrogel formulations were used to perform in vitro and in vivo experiments after 48 h of preparation. The various hydrogel formulations employed in this study are listed in Table 1.

2.3. Viscosity determination

Viscosity was determined in a cone and plate viscometer (Model DV-2, Brookfield Co., USA). A 0.5 g of cream or hydrogel was placed in the sample cup of the viscometer and allowed to stand to reach 37 °C. Viscosity measurements

<table>
<thead>
<tr>
<th>Composition</th>
<th>Capsaicin</th>
<th>Nonivarnid Pluronic</th>
<th>Chitosan</th>
<th>CMC-NH₂</th>
<th>CMC-Na</th>
<th>Ethanol</th>
<th>PG</th>
<th>Lactic acid</th>
<th>pH 4 buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>0.025</td>
<td>20</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Add to 100</td>
</tr>
<tr>
<td>F2</td>
<td>0.025</td>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Add to 100</td>
</tr>
<tr>
<td>F3</td>
<td>0.025</td>
<td>30</td>
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<td></td>
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<td></td>
<td></td>
<td>Add to 100</td>
</tr>
<tr>
<td>F4</td>
<td>0.025</td>
<td>2</td>
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<td></td>
<td></td>
<td></td>
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<tr>
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<td>0.025</td>
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<tr>
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<td>0.025</td>
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<tr>
<td>F8</td>
<td>0.025</td>
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<td>Add to 100</td>
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<tr>
<td>F9</td>
<td>0.025</td>
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<td>Add to 100</td>
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<tr>
<td>F10</td>
<td>0.025</td>
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<td>Add to 100</td>
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<td>F11</td>
<td>0.025</td>
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<tr>
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<td>0.025</td>
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<td>Add to 100</td>
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<tr>
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<td>0.025</td>
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<td>Add to 100</td>
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<tr>
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<tr>
<td>F15</td>
<td>0.075</td>
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<tr>
<td>F16</td>
<td></td>
<td>0.025</td>
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<td>Add to 100</td>
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<tr>
<td>F17</td>
<td></td>
<td>0.075</td>
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<td>Add to 100</td>
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</tbody>
</table>
were recorded after 60 s to obtain stable display reading.

2.4. In vitro skin permeation

The in vitro permeation experiments were determined by using Franz diffusion cell. The dorsal skin of female nude mouse (8–9 weeks old) was mounted on the receptor compartment with the SC-side facing upwards into the donor compartment and the dermal side facing downwards into the receptor. A 10 ml of 1:1 (v/v) ethanol-pH 7.4 citrate–phosphate buffer was used as the receptor medium. The donor compartment of the cell was filled with 1 g vehicle containing the test drug. The top of donor was covered with paraffin paper. The available diffusion area of cell was 0.785 cm². The receptor phase of cell was sustained at 37 °C and stirred by a magnetic stirrer at 600 rpm. At appropriate intervals, 200 μl aliquots of the receptor medium were withdrawn and immediately replaced by an equal volume of fresh receptor solution. Each formulation represents four experiments. The sample was analyzed by HPLC method described earlier (Fang et al., 1995).

The in vitro release rate experiment was determined by the same method as the in vitro permeation experiments except using cellulose membrane instead of the dorsal skin of female nude mouse.

2.5. Analytical method of capsaicin and nonivamide

The drug content of capsaicin was analyzed by a HPLC system consisting of a Hitachi L-7100 HPLC pump, a Hitachi L-7200 sample processor and a Hitachi L-7480 fluorescence detector. A 25 cm long, 4 mm inner diameter C18 column (LichroCart 250-4, Merck) was used. An automated integrator system (Hitachi L-7500) was used to determine the area under the curve (AUC). The mobile phase for capsaicin and nonivamide was 55% pH 4 citrate–phosphate buffer and 45% acetonitrile at a flow rate of 1.0 ml/min. The column effluent was passed through the fluorescence detector set at an excitation wavelength of 280 nm and an emission wavelength of 310 nm (Fang et al., 1995). The detection limit of capsaicin and nonivamide was 20 ng/ml.

2.6. In vivo evaluation

2.6.1. Subjects in the in vivo study

Six healthy male Chinese volunteers averaging 21.7 years of age (21–23 years) and with an average weight of 63.0 kg (52–70 kg) participated in this study. Written informed consents were obtained from all volunteers. None had any earlier or existing history of skin disease. The protocol was approved by the Ethical Committee of Mackay Memorial Hospital, Taiwan. The in vivo test of commercialized creams was performed first. After 1 week of wash-out period, the test of hydrogels was then examined.

2.6.2. Skin strippings

An accurately weighted 0.2 g of cream or hydrogel was spread uniformly over a sheet of cotton cloth (2 × 2 cm²). These pieces of cloth were applied on both volar forearms of volunteers (four pieces for each site) under an occlusive dressing for 1 or 4 h. After withdrawal of the residual base from the skin, a cotton wool swab immersed in methanol solution wiped off the remaining drug in skin surface before tests commenced. The SC was removed by nine strippings with adhesive tape (Transport®, 3M Co., USA). Immediately after stripping, the tape was stored in glass tube. A 5 ml of methanol was mixed with the nine strips in the glass tube and then followed by mechanical shaking for 1 h. After centrifugation for 5 min at 3000 rpm, the supernatant layer was withdrawn and directly injected into the HPLC.

2.6.3. Skin erythema determination

After the removal of SC, the level of erythema was determined at the appropriate intervals by Mexameter® (Model MX16, Courage and Khazaka Electronic Co., Germany). The probe of Mexameter® was placed on the treated skin surface. The measurement process automatically began and a spring in the probe provided constant pressure on the skin. The diameter of the measuring
surface was 5 mm. The result of erythema (Erythema index, Ei) was expressed as differences from the control, which was the value obtained from one adjacent untreated site. Therefore, changes in erythema level on the treated sites were measured against baseline standard of the volunteer.

The self pungent sensation was also determined. In this test the volunteers were asked to score the self pungent sensation induced by topical capsaicin and nonivamide application at the same intervals of skin erythema determination by Mexameter®.

3. Results and discussion

3.1. In vitro evaluations

3.1.1. In vitro permeation from commercialized creams

The cumulative amount-time profiles for various commercialized creams with capsaicin across nude mouse skin are shown in Fig. 1(A). The slopes of the resulting linear plots were computed, and the flux (µg/cm² per h) was calculated from the slopes. The curves of these creams were all suitable to fit using a zero-order equation. The flux of Zostrix® with 0.075% capsaicin was approximately threefold compared with that with 0.025% capsaicin. Similar result was observed in the capsaicin release rate across cellulose membrane (Fig. 1(B)). Both the flux and release rate of capsaicin from cream formulations increased in the order of 0.025% Zostrix® < 0.025% Zoscum® < 0.075% Capsaicin-C® < 0.075% Zostrix® (t-test, P < 0.05, Table 2). The transport of drug across cellulose membrane is via the pore within the membrane. While considering the structure of cellulose membrane, the molecular weight cut-off of this membrane is around 12,000–14,000. It suggests that there are channels for drug molecules to diffuse freely. Therefore, the penetration of the drug may predominantly depend on the release of drug from donor vehicle (Ho et al., 1994). As shown in Table 2, a linear relationship (correlation coefficient \( r = 0.96 \)) was observed between the permeation across cellulose membrane and skin from cream bases. This result may suggest that the diffusion of capsaicin through cream is an important mechanism controlling the whole skin permeation process.

3.1.2. In vitro permeation from Pluronic hydrogels

Various approaches have been used to improve the permeability and patient compliance of existing cream dosage forms. One of these successful approaches is the utilization of hydrogels prepared by polymers due to their controlled-release characterization, good tissue compatibility, easy manipulation of swelling level and, thereby, drug permeability (Kim et al., 1992). Pluronic is one of widely used polymer in drug delivery system. Its unique characteristics is reversible thermal gelation; concentrated solutions (20–30% w/w) of this polymer displayed fluid at refrigerator temperature, but become soft gels at body temperature (Barichello et al., 1999). The capsaicin permeation and release rate from 20, 25 and 30% Pluronic hydrogels (F1–F3) could also be described fairly well by a zero-order kinetic profile. Generally, the flux and release rate decreased as the concentration of Pluronic in the vehicle increased (Table 2). Pluronic hydrogels are viscous isotropic liquid crystals consisting of micelles. It is hypothesized that the drug is released by diffusion through the extramicellar water channels of the hydrogels matrix, and higher concentration of Pluronic causes smaller size of water channels, lower micellar growth rate, or greater tortuosity (Barichello et al., 1999; Shin et al., 1999). Pluronic hydrogels were shown to prevent capsaicin skin permeation as compared with commercialized creams. When the drug diffusion through the vehicle is a rate-limiting step, the viscosity of vehicles may play an important role in controlling the permeation of drug across the skin and should be determined (Ho et al., 1994). As depicted in Table 2, the viscosities of Pluronic hydrogels are over the detectable range of viscometer and greatly higher than those of creams. It is a general rule for increasing the viscosity of vehicles, which would cause a more rigid structure and decrease the rate of drug release.
3.1.3. In vitro permeation from chitosan hydrogels

Chitosan is an alkaline de-acetylated product derived from the living crustacean exoskeleton (Lu et al., 1999). This natural, cationic polysaccharide possesses great properties such as non-toxicity, high biocompatibility and non-antigenicity (Lu et al., 1999; Tsai et al., 1999). Although the chitosan hydrogels delivered 0.025% capsaicin across the skin in a zero-order fashion, the release of capsaicin across cellulose membrane followed Higuchi kinetics as its correlation coefficient ($r = 0.98–0.99$) predominated over zero-order kinetics ($r = 0.89–0.96$). This may indicate that the diffusion of capsaicin in chitosan matrix retards in the late stage of its dermapharmacokinetics. As the concentration of chitosan increases from 2 to 6% (F4–F6), the flux and release rate decrease significantly (Table...
This phenomenon is related to greater polymer entanglement and lower effective molecular diffusion area as chitosan concentration increases. A linear relationship \( r = 0.98 \) between capsaicin flux and release rate was also observed in chitosan hydrogels, indicating the importance of capsaicin diffusion in hydrogel matrix.

The capsaicin flux from chitosan hydrogels were all significantly higher \((t\text{-test, } P < 0.05)\) than from commercialized cream bases with 0.025% capsaicin. The viscosities of 4 and 6% chitosan bases were also significantly higher \((t\text{-test, } P < 0.05)\) than those of cream bases (Table 2). These results are contradictory to each other since the viscosity is always inversely proportional to the extent of permeation if diffusion through vehicle is the rate-limiting step (Ho et al., 1994). It consequently implied there was notable diffusion mechanism in vehicle involved in the skin permeation of capsaicin via chitosan hydrogels. Earlier study demonstrated that capsaicin was easily penetrated into the deeper layers of the skin via lipoidal pathway by partitioning from vehicle to the SC (Fang et al., 1995). The difference in the vehicle-SC partition would have contribution to the uptake of drug into the SC. As chitosan hydrogel is more hydrophilic than oil-in-water cream, the partition coefficient of lipophilic capsaicin would be expected to favor the SC. This theory can also partly explain the lower permeation of Pluronic hydrogels as compared with chitosan hydrogels due to the smaller amount of water in Pluronic formulations, resulting in the higher vehicle-SC partition of capsaicin than chitosan hydrogels.

### 3.1.4. In vitro permeation from CMC hydrogels

The anionic CMC is one of the synthetic water-soluble cellulose largely used as matrix for drug delivery systems (Doelker, 1987). The permeation of capsaicin from CMC with different counterions of \(-\text{NH}\text{\textsuperscript{+}} \text{(F7)}\) and \(-\text{Na}\text{\textsuperscript{+}} \text{(F9)}\) was compared. Similarly to chitosan hydrogels, the release of capsaicin from CMC hydrogels appeared to follow Higuchi kinetics \( (r = 0.98–0.99) \). The caps-

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Viscosity ((\text{cps} \times 10^3))</th>
<th>Flux ((\mu\text{g/cm}^2/\text{h} \text{ or } \mu\text{g/cm}^2 \text{ per h}^{1/2}))</th>
<th>Release rate ((\mu\text{g/cm}^2/\text{h} \text{ or } \mu\text{g/cm}^2 \text{ per h}^{1/2}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.025% Zostrix®</td>
<td>7.92 ± 0.56</td>
<td>1.09 ± 0.14</td>
<td>2.25 ± 0.09</td>
</tr>
<tr>
<td>0.075% Zostrix®</td>
<td>8.92 ± 1.90</td>
<td>3.56 ± 0.87</td>
<td>7.26 ± 0.46</td>
</tr>
<tr>
<td>0.025% Zoscum®</td>
<td>21.33 ± 0.90</td>
<td>1.64 ± 0.11</td>
<td>2.75 ± 0.29</td>
</tr>
<tr>
<td>0.075%</td>
<td>11.27 ± 0.90</td>
<td>2.52 ± 0.14</td>
<td>3.91 ± 0.32</td>
</tr>
<tr>
<td>Capsaicin-e®</td>
<td></td>
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</tr>
<tr>
<td>Fl</td>
<td>&gt; 102.00</td>
<td>0.06 ± 0.01</td>
<td>1.64 ± 0.23</td>
</tr>
<tr>
<td>F2</td>
<td>&gt; 102.00</td>
<td>0.03 ± 0.01</td>
<td>1.69 ± 0.07</td>
</tr>
<tr>
<td>F3</td>
<td>&gt; 102.00</td>
<td>0.02 ± 0.01</td>
<td>1.04 ± 0.06</td>
</tr>
<tr>
<td>F4</td>
<td>1.50 ± 0.06</td>
<td>4.90 ± 0.65</td>
<td>32.89 ± 3.92*</td>
</tr>
<tr>
<td>F5</td>
<td>24.40 ± 0.40</td>
<td>2.89 ± 0.24</td>
<td>22.50 ± 3.93*</td>
</tr>
<tr>
<td>F6</td>
<td>69.13 ± 1.80</td>
<td>2.53 ± 0.36</td>
<td>16.68 ± 2.34*</td>
</tr>
<tr>
<td>F7</td>
<td>&gt; 102.00</td>
<td>3.88 ± 0.10</td>
<td>27.96 ± 1.65*</td>
</tr>
<tr>
<td>F8</td>
<td>1.13 ± 0.11</td>
<td>6.79 ± 0.72</td>
<td>39.78 ± 4.21*</td>
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<tr>
<td>F9</td>
<td>40.67 ± 0.31</td>
<td>4.69 ± 0.20</td>
<td>21.28 ± 2.11*</td>
</tr>
<tr>
<td>F10</td>
<td>&gt; 102.00</td>
<td>2.17 ± 0.33</td>
<td>23.87 ± 5.63*</td>
</tr>
<tr>
<td>F11</td>
<td>53.20 ± 0.53</td>
<td>3.37 ± 0.49</td>
<td>25.93 ± 2.83*</td>
</tr>
<tr>
<td>F12</td>
<td>28.20 ± 13.46</td>
<td>2.37 ± 0.13</td>
<td>23.65 ± 3.58*</td>
</tr>
<tr>
<td>F13</td>
<td>94.60 ± 4.72</td>
<td>3.42 ± 0.44</td>
<td>30.13 ± 5.74*</td>
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<tr>
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<td>&gt; 102.00</td>
<td>2.36 ± 0.24</td>
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<tr>
<td>F15</td>
<td>40.67 ± 0.31</td>
<td>4.63 ± 0.59</td>
<td>12.69 ± 0.58</td>
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<tr>
<td>F16</td>
<td>40.67 ± 0.31</td>
<td>14.32 ± 147*</td>
<td>12.41 ± 1.10</td>
</tr>
<tr>
<td>F17</td>
<td>40.67 ± 0.31</td>
<td>18.14 ± 3.46*</td>
<td>21.12 ± 0.60</td>
</tr>
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</table>

Each value represents the mean ± S.D. \((n = 4)\).

* Higuchi kinetic model.
icin release rate across cellulose membrane from CMC-Na was significantly lower (t-test, \( P < 0.05 \)) than that from CMC-NH\(_4\) (Table 2). However, an opposite result was observed in flux across skin. It may be due to the good bioadhesion of CMC-Na to the skin, as the adhesive properties prolong the time of location at the site of application and improve permeation efficacy (Doelker, 1987; Jones et al., 1997). The viscosity of hydrogels was found to be higher as the amount of polymer increased (F8–F10, Table 2). High vehicle viscosity retarded capsaicin permeation, resulting from the increased difficulty to permeate from more sticking cellulose matrix (Lee et al., 1995). The fluxes of capsaicin from CMC-Na hydrogels were generally higher than that from the other creams and hydrogels. The result indicated that the hydrophilic characteristics of natural polymers, CMC-Na, resulted in the ease of capsaicin partitioning from hydrogel matrix to skin (Doelker, 1987; Narasimhan and Peppas, 1997).

In order to overcome the biological deficiencies of synthetic polymers and to enhance the mechanical characteristics of natural polymers, CMC-Na was blended with chitosan in different ratios (F11 and F12). The addition of CMC-Na into chitosan hydrogels reduced the flux of capsaicin (F5 vs. F12, Table 2). The same phenomenon was observed in the addition of chitosan into CMC-Na hydrogels (F9 vs. F11, Table 2). Chitosan may interact with CMC-Na to form water-insoluble complex and compact structure (Lin and Lin, 1992), which may be responsible for the higher viscosity of binary hydrogels than single polymer hydrogels (Table 2).

The solubilizing agents-propylene glycol (PG) and ethanol were incorporated into the CMC-Na hydrogels (F13 and F14). Adding these hydrophobic organic solvents as solubilizing agents reduced the polarity of hydrogel. The permeation of capsaicin across skin consequently decreased by PG and ethanol as shown in Table 2. Capsaicin molecules would incorporate into solubilizing agent-added hydrogels so as to get a stronger affinity than that of hydrophilic non-solubilizing agent gel base. No significant difference (t-test, \( P < 0.05 \)) was observed between the release rate of PG-added hydrogels and non-solubilizing agent hydrogels. Additionally, ethanol showed lesser inhibition effect on the release rate of capsaicin than its flux across skin (Table 2). This indicated that the influence of unknown factors other than the affinity between hydrogel and capsaicin predominated in the flux of capsaicin transported from solubilizing agent-added hydrogels across the skin. Both PG and ethanol have been shown to have skin barrier-altering properties (Aspe et al., 1995). Changes in skin structure induced by PG and ethanol could reduce the permeation of drug across skin (Inagi et al., 1981; Fang et al., 1999).

The release rate of capsaicin from CMC-Na hydrogels with higher dose (0.075%, F15) was significantly higher than that with lower dose (0.025%, F8) (Fig. 2(B), Table 2), but the flux across skin of both was approximately equal. Although higher dose of capsaicin can diffuse through CMC-Na hydrogels with higher rate, the skin reservoir of capsaicin may be fully saturated, contributing to the retardation of entrance of medication into the already saturated skin. This dose-dependent phenomenon was not observed in Zostrix\textsuperscript{®} creams since the skin reservoir had never reach the saturation status with the low degree of diffusion (Fig. 1).

The release rate of nonivamide from CMC-Na hydrogels (F16 and F17) is significantly higher (t-test, \( P < 0.05 \)) than that of capsaicin both in the 0.025 and 0.075% doses (Fig. 2(B)). Interestingly, the permeation of nonivamide across the skin leveled off at the late stage of in vitro topical application (10–24 h). The cumulative amount of nonivamide at 24 h is even lower (t-test, \( P < 0.05 \)) than that of capsaicin (Fig. 2(A)). These evidences might suggest the smaller amount of nonivamide was needed to saturate the skin reservoir than that of capsaicin. Another explanation was that nonivamide was more hydrophilic than capsaicin according to n-octanol/water partition coefficient (Tsai et al., 1994), resulting in the poorer ability of nonivamide partitioning into the skin.

3.2. In vivo evaluations

3.2.1. Skin strippings

The commercialized creams and the CMC-Na hydrogels containing 0.025 and 0.075% capsaicin would incorporate into solubilizing agent-added hydrogels so as to get a stronger affinity than that of hydrophilic non-solubilizing agent gel base. No significant difference (t-test, \( P < 0.05 \)) was observed between the release rate of PG-added hydrogels and non-solubilizing agent hydrogels.
Fig. 2. Cumulative amount of capsaicin and nonivamide detected in the receptor compartment versus time plots from CMC-Na hydrogels across (A) nude mouse skin; and (B) cellulose membrane. 0.025% capsaicin (●); 0.075% capsaicin (○); 0.025% nonivamide (▼); 0.075% nonivamide (▼). All data represent the means of four experiments ± S.D.

and nonivamide (F9, F15–F17) were chosen to study the following in vivo evaluations. Cumulative amounts of capsaicin and nonivamide in SC was detected at 1 and 4 h after topical application in humans by modifying FDA-approved skin stripping method (Shah et al., 1998). As shown in Fig. 3(A), the cumulative amount of capsaicin after 4 h application is higher than 1 h application from all cream bases although there is no significant difference (t-test, P < 0.05) between 1 and 4 h amount in 0.075% Zostrix® and Zoscum®. The cumulative amount of 0.025% Zostrix® at 4 h was approximate to that of 0.075% Zostrix® at 1 h, indicating more concentrated cream could achieve same degree of permeation in a shorter period of application. The in vivo skin stripplings and in vitro permeation showed a same trend of 0.025% Zostrix® < Zoscum® < 0.075% Zostrix® (r = 0.90
at 1 h, \( r = 0.99 \) at 4 h, Fig. 1(A) and Fig. 3(A)), suggesting poor permeation of Capsaicin-C® cream in the in vivo status.

The 1 h cumulative amount of CMC-Na hydrogels with 0.025% capsaicin (F9) was significantly higher (\( t \)-test, \( P < 0.05 \)) than that of 0.025% Zostrix® (Fig. 3(B)). However, no significant difference (ANOVA test, \( P > 0.05 \)) was detected among the 1 and 4 h cumulative amount of CMC-Na hydrogels, as well as 4 h cumulative amount of 0.025% Zostrix®. The 1 and 4 h cumulative amounts from CMC-Na hydrogel with 0.075% capsaicin (F15) were also close to the values with 0.025% (F9). These results demonstrated that CMC-Na provided a higher permeation of capsaicin at the early stage of topical application, but did not last to the late stage of application after saturating drug reservoir in SC. This theory also explained the in vitro permeation of CMC-Na hydrogels.

Similar to the results of in vitro skin permeation, the 1 h cumulative amount of nonivamide in SC in the in vivo test was lower than that of capsaicin without significant difference (F16 vs.

Fig. 3. Cumulative amount of capsaicin and nonivamide in the stratum corneum by in vivo skin stripping technique from (A) commercial creams; and (B) CMC-Na hydrogels. All data represent the means of six experiments ± S.D.
Fig. 4. Skin erythema level versus time plots after 1 h topical application of capsaicin commercial creams on volar forearms of six subjects (volunteer A–F). 0.025% Zostrix® (●); 0.075% Zostrix® (○); 0.025% Zoscam® (▼); 0.075% Capsaicin-C® (▲).

3.2.2. Skin erythema determination

The skin erythema after 1 and 4 h topical application of capsaicin creams is shown in Fig. 4 and Fig. 5, respectively. The placebo group showed that Ei values were all near zero through the detection duration (data not shown). A wide inter-subject variation in erythema response was observed. This variation can probably be explained by true biological differences in skin susceptibility to irritants between different subjects.
All volunteers showed the peak Ei (Ei_{max}) values within 1 h after topical application of cream for 1 h (Fig. 4). The values of Ei then gradually leveled off after 2 h. The therapeutic effects of capsaicin are subsequent to initial nerve stimulation which evokes erythema and the sensation of pungent, which can be observed clearly at the beginning of application. The initial erythema and pain progressively decrease since capsaicin prevents neuropeptide re-accumulation (Simone and Ochoa, 1991). Hence the skin underwent the early irritative to late depressive phenomenon after topical application of capsaicin as depicted in Fig. 4. The

Fig. 5. Skin erythema level versus time plots after 4 h topical application of capsaicin commercial creams on volar forearms of six subjects (volunteer A–F). 0.025% Zostrix® (●); 0.075% Zostrix® (○); 0.025% Zoscum® (▼); 0.075% Capsaicin-C® (▼).
late depressive phenomenon could partially be explained by the theory that capsaicin was desorbing from the skin until withdrawing cream bases and depleting the drug reservoir. Qualitatively, the pungent sensation determinations by all volunteers showed the maximum sensation occurred at 0 or 0.5 h after removing capsaicin creams. The self-reported pungent sensation had occurred before the skin erythema. No pungent sensation was determined from all subjects after 1.5 h of withdrawing creams.

The $E_{\text{max}}$ value of 0.075% Zostrix® was higher than of 0.025% Zostrix® in all volunteers, demonstrating cutaneous dose-dependent response (Fig. 4). The same result was reported by pungent sensation recorded by subjects. The result could

Fig. 6. Skin erythema level versus time plots after 1 h topical application of capsaicin and nonivamide hydrogels on volar forearms of six subjects (volunteer A–F). 0.025% capsaicin (●); 0.075% capsaicin (○); 0.025% nonivamide (▲); 0.075% nonivamide (▽).
Fig. 7. Skin erythema level versus time plots after 4 h topical application of capsaicin and nonivamide hydrogels on volar forearms of six subjects (volunteer A–F). 0.025% capsaicin (●); 0.075% capsaicin (○); 0.025% nonivamide (▼); 0.075% nonivamide (▼). be due to the higher capsaicin absorption of 0.075% Zostrix® as observed in the in vitro skin permeation and in vivo skin strippings (Fig. 1(A) and Fig. 3(A)). The higher capsaicin absorption of Zoscum® than 0.025% Zostrix® in the in vitro and in vivo tests also contributed to the greater $E_{\text{max}}$ value of Zoscum® than 0.025% Zostrix® in all volunteers. Capsaicin-C® and 0.025% Zostrix® showed the lowest $E_{\text{max}}$ value in two-third volunteers, respectively, indicating the low capsaicin absorption of these two formulation.

The skin erythema returned to normal condition within 1–1.5 h after the removal of capsaicin creams at 4 h application (Fig. 5). The difference of $E_{i}$ data between 0.025 and 0.075% Zostrix® was roughly. The same phenomenon was ob-
served after comparing 0.025% Zostrix® and Zoscum® creams since three volunteers had higher Eimax values in 0.025% Zostrix® and the other three volunteers showed the opposite result. This indicates that it is not necessary for capsaicin to depend on applying dose and formulation after longer administered duration.

The 1 h application of CMC-Na hydrogels with capsaicin and nonivamide (F9, F15–F17) on the skin of subjects reached its higher level within 0.5 h except volunteers D as shown in Fig. 6. Skin erythema then decreased to normal skin condition after 2.5–3 h of withdrawing hydrogels. All subjects reported stronger pungent sensation of CMC-Na hydrogels than cream bases. Compared with cream bases, CMC-Na hydrogels may provide a rapid onset and prolonged duration of capsaicin release. In general, the Ei max value of CMC-Na hydrogels with 0.025% capsaicin (F9) was higher than that of 0.025% Zostrix®. On the other hand, the Ei values of hydrogels with 0.075% capsaicin (F15) approached to the values of 0.025% capsaicin (F9). The in vitro skin permeation and in vivo cumulative amount in SC at 1 h paralleled to skin erythema induced by these formulations (Table 2 and Fig. 3).

The similar cumulative amounts of capsaicin in SC after 1 h application of 0.025 and 0.075% nonivamide contributed to the close skin erythema in five-sixth subjects (Figs. 3 and 6). Although the amount of nonivamide in SC was lower than that of capsaicin after 1 h application of hydrogels, the Ei max value showed an opposite result both in 0.025 and 0.075% doses. This suggested that nonivamide in hydrogels induced higher erythema on human skin than capsaicin. It was difficult for the volunteers to distinguish the degrees of pungent sensation of four CMC-Na hydrogels with various doses of capsaicin and nonivamide. The majority of subjects reported a longer sensation in nonivamide-treated sites than in capsaicin-treated sites.

The one-third subjects showed similar degrees of the skin erythema between two doses of capsaicin (Fig. 7). The Ei max value of 0.025% Zostrix® was generally higher than that of hydrogels with 0.025% capsaicin (F9) after 4 h application which might be due to the retardation of capsaicin absorption in SC at the late stage of topical application. Skin erythema in the nonivamide-treated skin reached its maximum at 0.5 h after removing hydrogels at 4 h in all subjects. No clear association could be found between the degrees of erythema with low dose and high dose nonivamide (F16 and F17, Fig. 7). As compared with the erythema level of nonivamide and capsaicin after 4 h application, most of the subjects (two-third) exhibited higher Ei max values in both of 0.025 and 0.075% nonivamide. The remaining one-third subjects showed similar Ei max value between capsaicin- and nonivamide-treated skin. It may suggest the stronger skin erythema of nonivamide than capsaicin after both short and long administered duration.

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