Evaluation of Percutaneous Absorption of Midazolam by Terpenes

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Summary: Midazolam is a highly lipophilic drug that is widely used in preanesthetic medication. Recently, terpenes have been reported to show an enhancing effect on percutaneous absorption of drugs. The effect of terpenes (l-menthol, d-limonene, RS-(±)-ß-citronellol, geraniol) on the in vitro percutaneous absorption of midazolam through rat skin was evaluated using unjacketed Franz diffusion cells. Since midazolam is a lipophilic drug, percutaneous penetration is low and a percutaneous penetration enhancer is necessary for its percutaneous absorption. The terpenes (5%, w/v) in combination with 30% ethanol, and 20% propylene glycol significantly increased the percutaneous absorption of midazolam in comparison to the control. In vitro data suggested that d-limonene is the most effective enhancer among terpenes and other penetration enhancers such as Azone. In in vivo percutaneous absorption assays, the midazolam formulation using d-limonene could penetrate through rat skin, but the other terpenes could not penetrate. In conclusion, d-limonene in combination with ethanol can be used to enhance the percutaneous absorption of the highly lipophilic drug midazolam.

Key words: percutaneous absorption; penetration enhancers; terpenes; midazolam

Introduction
Midazolam, a short-acting benzodiazepine derivative, is used for preanesthetic sedation medication in children.1,2 Respiratory depression associated with midazolam occurs much less frequently in comparison with other benzodiazepines.3 Midazolam is administered by an intravenous or intramuscular injection.4 However, neither route can avoid pain stress and alternative administration routes, such as rectal,5 nasal,6 and sublingual,7 have been proposed to solve this problem of patients' stress induced by injection.7 In this study, we investigated a formulation for simple, non-invasive, transdermal administration of midazolam. Midazolam is a highly lipophilic drug (log P = 2.68) with low percutaneous absorption,10 and therefore a percutaneous absorption enhancer is needed to develop midazolam formulation that can be adsorbed percutaneously. To evaluate the percutaneous absorption of midazolam, Touitou studied the in vitro skin permeation profiles of midazolam through hairless mouse skin from various solvent, and Azone® increases the permeation flux.11 Azone® is a pharmacologically active, irritant, and damaging to the skin, however, a low toxic percutaneous enhancer is acceptable for transdermal delivery pharmaceutics. Terpenes, constituents of essential oils, have low toxicity and have been reported to show enhancing effects on the percutaneous absorption of drugs.12–15 Terpenes are relatively safe, clinically acceptable enhancers for use with lipophilic drugs.13 d-limonene and l-menthol, monocular monoterpene, enhance the penetration of diazepam through rat skin.16–18 In this study, ethanol and hydroxypropyl cellulose (HPC), a gelling agent, were incorporated into gel formulations in order to develop percutaneous formulation of midazolam. Ethanol was found to affect terpene enhancing activity. HPC belongs to a family of inactive hydrophobic non-ionic polymers widely used in oral pharmaceutical agents. In topical formulations, such as a topical gel, HPC polymers are used as suspending and stabilizing agents.19 The purpose of this study was to evaluate the enhancing effects of four natural terpenes (l-menthol, d-limonene, RS-(±)-ß-citronellol, and geraniol) to investigate their effects on the in vitro and in vivo percutaneous absorption of midazolam from HPC gels containing ethanol and propylene glycol.

Materials and methods
1. Materials: Midazolam was supplied kindly from Yamanouchi Pharmaceutical Co., Ltd (Tokyo, Japan). RS-(±)-ß-citronellol was purchased from Aldrich Chemical Co. Inc (Milwaukee, WI, USA).
l-menthol and geraniol were purchased from Katayama Chemical Co., Ltd. (Tokyo, Japan). d-limonene was purchased from Wako Junyaku Kogyo. (Osaka, Japan). Azone was purchased from Atlantic Microlabs (Atlanta, GA, USA). HPC was purchased from Tokyo Kasei Co., Ltd. (Tokyo, Japan). All other reagents used were of analytical grade.

2. Preparation of epidermis: Male Wistar rats weighing 230 to 280 g were used in the in vitro permeation and in vivo percutaneous absorption studies. The experiments were performed in a constant temperature room (25 ± 2°C). On the day before the experiment, the abdominal hair was carefully removed with electric clippers without breaking the skin. Epidermal membranes were prepared by a heat separation technique. The whole skin was soaked in water at 60°C for 45 s, followed by careful removal of the epidermis. The epidermis was washed with water and used in the in vitro percutaneous absorption studies.

3. Solubility of midazolam: The solubility of midazolam was determined by agitating excess solute in the control or enhancer solutions for 24 h at 37°C, and then determining the amount of midazolam in the saturated solution by high performance liquid chromatography (HPLC).

4. Preparation of midazolam formulation: The procedure for the preparation of the midazolam formulation was previously described. This formulation was composed of midazolam (5 mg), HPC (0 or 3%), ethanol (0 or 30%), propylene glycol (0 or 20%), terpenes (5%), and water. For the preparation of the midazolam formulation, ethanol was mixed with distilled water and propylene glycol using a mixer. HPC was added slowly until a clear gel was formed. Midazolam was not completely soluble in the formulation but was in the form of a suspension.

5. In vitro percutaneous absorption: Franz (vertical) diffusion cells were used in the in vitro percutaneous absorption studies. The epidermis was sandwiched between the cells with the stratum corneum facing the donor compartment. The surface area of the epidermis exposed to the solution was 3.14 cm². The donor compartment contained 1 mL of the midazolam formulation and the receiver compartment contained 18 mL of saline, pH 7.4. Thus, midazolam donor was at a saturating concentration. The donor compartment was capp with a glass cap that highly fit the neck of the donor compartment to prevent evaporation of the solvents. The cells were maintained at 37 ± 0.5°C. The contents of the receiver compartment were stirred with a magnetic bar at 100 rpm. At specified intervals, 0.5 mL samples were withdrawn from the receiver compartment, and an equivalent amount of saline (0.5 mL) was added to maintain a constant volume. Control experiments were also performed using 30% ethanol and/or 20% propylene glycol and/or 3% HPC in distilled water without terpenes. All the experiments were run for 12 h. The samples were assayed for midazolam content by HPLC.

6. In vivo absorption study: The animals were fasted for 12 h before the experiment and water was supplied ad libitum. In the transdermal permeation study, the rats were fixed supinely and the abdominal hair was removed with electric clippers. Teflon cells (surface area 3.14 cm²) were fixed to the rat body using polyacrylate glue. The formulation was applied to the Teflon ring component. The rats were anesthetized with an intraperitoneal injection of urethane, 1 g/kg, and the jugular vein was cannulated to facilitate removal of blood samples. The midazolam formulation (1 mL) was applied to the Franz cells (surface area 3.14 cm²) on the abdominal skin. At intervals after the drug administration, a blood sample (0.3 mL) was collected from the jugular vein and centrifuged at 3,000 rpm for 10 min. The plasma concentration of drug was determined by HPLC. The area under the plasma concentration curve (AUC) up to 12 h post-administration was calculated by the trapezoidal method.

7. Midazolam HPLC determination: The chromatographic system consisted of an LC-6A pump (Shimadzu) and a variable wavelength UV detector, SPD-6AV (Shimadzu). Midazolam was analyzed by isocratic reversed-phase HPLC using a LiChrospher RP18 (5 μm, 250 × 4 mm, Merck Co., USA) with a mobile phase of 10 mM phosphate buffer (pH 7.0): acetonitrile (58:42) at a 1 mL/min flow rate and a 256 nm detection wavelength at ambient temperature. The injection volume was 50 μL and the midazolam concentration range for calibration was from 50 to 1,000 ng/mL. The midazolam retention time was 15 min.

8. Data analysis: The permeation of midazolam from the solvent mixture was measured over 12 h and plots were constructed of the cumulative corrected amounts of midazolam (μg/cm²) against time (h). The x-intercept of the extrapolated linear region gave the lag time. The slope of this linear portion of the graph provided maximum flux values at the steady state (Flux, μg/cm²/h). The permeation-enhancing activities were expressed as enhancement ratios of flux (ERflux), i.e. the ratio of the flux value with enhancer to that obtained with control.

\[
ER_{flux} = \frac{\text{Midazolam flux with percutaneous enhancer}}{\text{Midazolam flux without percutaneous enhancer}}
\]
(without terpene).23

Statistical analysis was performed using analysis of variance (ANOVA) and Duncan’s multiple range test. The level of significance was taken as \( P<0.05 \). The data are expressed as mean±standard error.

Result and Discussion

**In vitro** percutaneous absorption: The effect of terpenes (\( l \)-menthol, \( d \)-limonene, RS-(\( \pm \))-\( \beta \)-citronellol, geraniol) and Azone* on the **in vitro** percutaneous absorption profiles of midazolam through rat skin in distilled water are shown in Fig. 1. Table 1 summarizes the data on the percutaneous penetration and physicochemical parameters (Flux, ER\(_{sol} \), lag time, solubility and ER\(_{sol} \)) of midazolam in distilled water, 20% propylene glycol, 20% propylene glycol/30% ethanol, and 3% HPC/20% propylene glycol/30% ethanol. The solubility of midazolam was enhanced by terpenes, especially geraniol (\( p<0.01 \)). The solubility of midazolam in the solvent may contribute to increasing the percutaneous penetration. The flux for geraniol was significantly higher than that of the other terpenes in distilled water. Furthermore, to increase the solubility

### Table 1. Flux, ER\(_{sol} \), lag time, solubility and ER\(_{sol} \) of midazolam with or without enhancers

<table>
<thead>
<tr>
<th>(1) Distilled water (suspension, non-gel)</th>
<th>Flux (( \mu g/cm^2/hr ))</th>
<th>ER(_{sol} )</th>
<th>Lag time (hr)</th>
<th>Solubility (( \mu g/mL ))</th>
<th>ER(_{sol} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.15 ± 0.01</td>
<td>—</td>
<td>14.18 ± 1.82*</td>
<td>75.0 ± 12.9</td>
<td>—</td>
</tr>
<tr>
<td>( l )-menthol</td>
<td>0.19 ± 0.12</td>
<td>1.27</td>
<td>8.67 ± 0.01</td>
<td>117.5 ± 22.7</td>
<td>1.57</td>
</tr>
<tr>
<td>( d )-limonene</td>
<td>0.27 ± 0.06</td>
<td>1.8</td>
<td>5.05 ± 0.06*</td>
<td>218.7 ± 60.4*</td>
<td>2.92</td>
</tr>
<tr>
<td>RS-(( \pm ))-( \beta )-citronellol</td>
<td>0.23 ± 0.04</td>
<td>1.53</td>
<td>3.83 ± 1.14*</td>
<td>148.2 ± 30.1</td>
<td>1.98</td>
</tr>
<tr>
<td>Geraniol</td>
<td>0.34 ± 0.06</td>
<td>2.27</td>
<td>2.09 ± 0.45*</td>
<td>703.5 ± 83.3**</td>
<td>9.38</td>
</tr>
<tr>
<td>Azone*</td>
<td>0.20 ± 0.02</td>
<td>1.33</td>
<td>6.52 ± 1.45*</td>
<td>90.0 ± 13.2</td>
<td>1.2</td>
</tr>
</tbody>
</table>

(2) 20% Propylene glycol (suspension, non-gel)

<table>
<thead>
<tr>
<th>Control</th>
<th>0.17 ± 0.03</th>
<th>0.79 ± 0.06</th>
<th>1156.6 ± 211.8</th>
<th>—</th>
</tr>
</thead>
<tbody>
<tr>
<td>( l )-menthol</td>
<td>0.66 ± 0.13</td>
<td>3.88</td>
<td>2.77 ± 0.59*</td>
<td>791.2 ± 135.4*</td>
</tr>
<tr>
<td>( d )-limonene</td>
<td>1.92 ± 0.28**</td>
<td>11.29</td>
<td>2.09 ± 0.46</td>
<td>576.8 ± 129.8*</td>
</tr>
<tr>
<td>RS-(( \pm ))-( \beta )-citronellol</td>
<td>0.97 ± 0.08*</td>
<td>5.71</td>
<td>4.25 ± 0.13**</td>
<td>447.9 ± 131.7**</td>
</tr>
<tr>
<td>Geraniol</td>
<td>1.04 ± 0.01*</td>
<td>6.12</td>
<td>3.94 ± 0.05*</td>
<td>1108.8 ± 171.2</td>
</tr>
<tr>
<td>Azone*</td>
<td>2.05 ± 0.05**</td>
<td>12.06</td>
<td>4.96 ± 0.03**</td>
<td>684.9 ± 119.9*</td>
</tr>
</tbody>
</table>

(3) 20% Propylene glycol/30% Ethanol (suspension, non-gel)

<table>
<thead>
<tr>
<th>Control</th>
<th>0.92 ± 0.07</th>
<th>—</th>
<th>4.02 ± 0.13</th>
<th>1664.8 ± 148.5</th>
<th>—</th>
</tr>
</thead>
<tbody>
<tr>
<td>( l )-menthol</td>
<td>6.70 ± 1.02</td>
<td>7.28</td>
<td>1.25 ± 0.46*</td>
<td>1398.6 ± 157.7</td>
<td>0.84</td>
</tr>
<tr>
<td>( d )-limonene</td>
<td>56.02 ± 2.75**</td>
<td>60.89</td>
<td>3.38 ± 0.26</td>
<td>1372.8 ± 161.2</td>
<td>0.82</td>
</tr>
<tr>
<td>RS-(( \pm ))-( \beta )-citronellol</td>
<td>5.04 ± 0.37</td>
<td>5.48</td>
<td>2.19 ± 0.72</td>
<td>1215.5 ± 152.7</td>
<td>0.73</td>
</tr>
<tr>
<td>Geraniol</td>
<td>3.88 ± 0.36</td>
<td>4.22</td>
<td>2.31 ± 0.46</td>
<td>1254.8 ± 130.1</td>
<td>0.75</td>
</tr>
<tr>
<td>Azone*</td>
<td>11.71 ± 1.64*</td>
<td>12.72</td>
<td>3.05 ± 0.01</td>
<td>720.7 ± 162.4*</td>
<td>0.43</td>
</tr>
</tbody>
</table>

(4) 3% HPC/20% Propylene glycol/30% Ethanol (gel)

<table>
<thead>
<tr>
<th>Control</th>
<th>0.51 ± 0.07</th>
<th>—</th>
<th>3.50 ± 0.23</th>
<th>1664.8 ± 148.5</th>
<th>—</th>
</tr>
</thead>
<tbody>
<tr>
<td>( l )-menthol</td>
<td>4.47 ± 1.37</td>
<td>8.76</td>
<td>1.70 ± 0.44</td>
<td>1398.6 ± 157.7</td>
<td>0.84</td>
</tr>
<tr>
<td>( d )-limonene</td>
<td>30.48 ± 9.13*</td>
<td>59.76</td>
<td>3.86 ± 0.47</td>
<td>1372.8 ± 161.2</td>
<td>0.82</td>
</tr>
<tr>
<td>RS-(( \pm ))-( \beta )-citronellol</td>
<td>6.10 ± 1.63</td>
<td>11.96</td>
<td>2.88 ± 0.92</td>
<td>1215.5 ± 152.7</td>
<td>0.73</td>
</tr>
<tr>
<td>Geraniol</td>
<td>3.63 ± 0.55</td>
<td>7.12</td>
<td>2.56 ± 1.10</td>
<td>1254.8 ± 130.1</td>
<td>0.75</td>
</tr>
<tr>
<td>Azone*</td>
<td>8.57 ± 0.79</td>
<td>16.8</td>
<td>3.15 ± 0.43</td>
<td>720.7 ± 162.4*</td>
<td>0.43</td>
</tr>
</tbody>
</table>

Control: without enhancer

Each value represents mean±S.E.M. (n = 3)

\*\( P<0.05 \), \**\( P<0.01 \) compared with control.
of midazolam, 20% propylene glycol was added as a solubilizer. The in vitro percutaneous penetration of midazolam increased with elevations of its solubility. The order of the effectiveness of terpenes (Azone\textsuperscript{*} was positive control) for flux was Azone\textsuperscript{*} = d-limonene > geraniol = RS-(\textdaggerdbl)\textendash \textdagger)-\textbeta\textendash citronellol = l-menthol. Ethanol was added to provide solubilization and percutaneous penetration enhancing actions. The addition of 30% ethanol further increased the solubility of midazolam and improved the percutaneous absorption in comparison with 20% propylene glycol. In particular, d-limonene, which does not have a hydroxyl group in its molecular structure, showed the strongest penetration effect ($P < 0.01$). These results indicate that d-limonene is the most effective terpene for enhancing the permeability of midazolam. On the basis of corrected enhancement factors, the order of the effectiveness of terpenes was d-limonene > Azone\textsuperscript{*} > l-menthol = RS-(\textdaggerdbl)\textendash \textbeta\textendash citronellol = geraniol. The effects of an enhancer on the permeation of a drug usually depend on the physiochemical characteristics of enhancer and drug. The present results suggest that the limonene, hydrophobic, without a hydroxyl group is effective in penetrating the skin penetration compared to hydrophilic terpenes containing functional groups with hydrogen-bonding ability. It was reported that d-limonene was effective in enhancing the transport of lipophilic molecules such as indomethacin\textsuperscript{24-25} and ketoprofen\textsuperscript{26,27}. The highest skin penetration of midazolam by d-limonene may be attributed to its lipophilic characteristic.

However, since midazolam and terpenes are not completely dissolved but form a suspension, it is thought that a dispersing component is required. We evaluated the effect of a gel on the percutaneous penetrating action and selected HPC as the gelling agent since it is a hydrophilic macromolecule with non-ionic properties. This formulation for percutaneous penetration is shown in Fig. 2. The penetration of midazolam was less in the gel than in the non-gel. The lag time did not change. From these results, it is considered that gel formulation did not affect the diffusivity of midazolam in the skin. On the other hand, propylene glycol or ethanol increased solubility of midazolam, but terpene decreased it. It is estimated that the physicochemical properties of base containing propylene glycol or ethanol vary with the addition of terpene. Terpenes may decrease the lipophilicity of base and the solubility of midazolam was dropped. Given the above-mentioned results, we further evaluated in vivo percutaneous absorption of the gel formulation in rats.

**In vivo percutaneous absorption:** The percutaneous absorption of the prepared formulation of midazolam in 5% terpenes in combination with 3% HPC, 30% ethanol and 20% propylene glycol was tested in rats and the results are shown in Fig. 3. Only the formulation containing d-limonene could be absorbed in rats, but the other terpenes and Azone\textsuperscript{*} could not penetrate the rat skin. The concentration of midazolam with d-limonene reached a steady state after 10 h and the C\textsubscript{max} was 76.3 ng/mL after 12 h. These results were similar to those in vitro. d-limonene was appeared to be the most effective agent as a percutaneous absorption enhancer for midazolam.

The percutaneous absorption properties of lipophilic drugs, such as midazolam are low. We attempted to develop a percutaneous absorption formulation using...
terpenes. Terpenes have been reported to show an enhancing effect on percutaneous absorption, although this effect is variable.\(^{26}\) Since the solubility of midazolam is low, propylene glycol and ethanol were needed as solvents. A synergetic effect with ethanol and propylene glycol was observed. Ethanol is widely used in percutaneous absorption pharmaceutical preparations such as nitroglycerin\(^{28}\) and estradiol.\(^{29}\) It is generally accepted that solvents such as ethanol or propylene glycol used with enhancers accumulate in the tissue, and increase the partitioning of drugs due to the large affinities of drugs for the solvents.\(^{30}\) In this study, midazolam showed effective percutaneous absorption both in vivo and in vitro. d-limonene in combination with ethanol showed the highest enhancing effect on the percutaneous adsorption of the highly lipophilic drug midazolam. These results provide useful information to develop a new dosage formulation for midazolam.

References


25) Levison, K. K., Takayama, K., Isowa, K., Okabe, K. and


