Keratinized Epithelial Transport of β-Blocking Agents. III. Evaluation of Enhancing Effect on Percutaneous Absorption Using Model Lipid Liposomes

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We investigated the usefulness of a liposome composed of lipid components of stratum corneum as a system to evaluate the enhancing ability of various penetration enhancers. Changes in the lipid fluidity of the model lipid liposome membrane, consisting of ceramide (40%), cholesterol (25%), palmitic acid (25%) and cholesterol 3-sulfate (10%), by the addition of 1-dodecylazacycloheptan-2-one (Azone), oleic acid (OA) or dimethyl sulfoxide (DMSO) were measured by a fluorescence polarization method using 1,6-diphenyl-1,3,5-hexatriene, dansylhexadecylamine and cholesterol anthracene-9-carboxylate as probes for the hydrophobic polar, the polar head and cholesterol regions of the lipid bilayer, respectively. The order of lipid fluidizing ability was suggested to be Azone > OA > DMSO. An in vitro permeation study of six β-blocking agents, propranolol, metoprolol, timolol, pindolol, nadolol and atenolol, was carried out to estimate the enhancing effect of the enhancers using rat abdominal skin. Azone showed a strong facilitating effect on the drug permeation and the effect of OA was weaker than that of Azone. DMSO showed little effect unless a high concentration of over 60% (w/w) was employed. The basis for these enhancing mechanisms is the change in diffusion constant of a drug in the rat skin.

These results were consistent with those obtained from the lipid fluidity measurement, that is, the stronger the increment effect of the enhancer on the fluidity of the model lipid liposome, the larger is the D value for the drug in the skin. Applicability of the model lipid liposome in evaluating the penetration enhancer was thus demonstrated.

Keywords enhancing effect; lipid membrane fluidity; liposome; model lipid; stratum corneum

The stratum corneum layer is known to be a major barrier to drug permeation in the keratinized epidermis. For most drugs, the major barrier function of the stratum corneum has been attributed to the intercellular lipids, mainly to their nature and their ordered multilayers.1-4 Consequently, in order to extend the variety of drugs that might be administered via the skin and also to increase the local and systemic activities of topically applied drugs, considerable attention has recently been focused on skin penetration enhancers.5,6 In the past, the most widely used penetration enhancers were the apotic solvents, such as dimethyl sulfoxide (DMSO), dimethylformamide and dimethylacetamide, and the pyridolones, 1-dodecylazacycloheptan-2-one (Azone), fatty acid, surfactants and alcohols. The mode of action of each of these enhancers differs, but most have been proved to interact in some way with the stratum corneum lipid structure, generally by increasing the fluidity of the intercellular lipid bilayers.6,7 To better understand the mechanism of action of various classes of penetration enhancers, multilamellar vesicles (MLV) of dipalmitoylphosphatidylcholine (DPPC) have been used as a simple model representing the lipid bilayers of the stratum corneum. Physical techniques of NMR, ESR, X-ray diffraction, raman spectroscopy and Fourier transform infrared (FTIR) spectroscopy have also been used to study the thermotropic behavior of the lipids.8-12 The purposes of this work were to establish a model lipid system of stratum corneum intercellular lipids for evaluating the enhancement effect of various substances, and to discuss the usefulness of the evaluation methods using the model lipids. Azone, oleic acid (OA) and DMSO were selected as model enhancers having different modes of action. The model lipid system evaluated was a MLV liposome, consisting of a mixture of ceramide, cholesterol, palmitic acid and cholesterol sulfate, and the effects of enhancers on the fluidity of the model lipid liposome membrane were evaluated by a fluorescence polarization method. Further, the relationship between the ability to change membrane fluidity and the enhancing effect of the enhancer is discussed.

Materials and Methods

Materials Propranolol hydrochloride was purchased from Nacalai Tesque Co. (Kyoto). Metoprolol tartrate and pindolol were obtained from Sigma Chemical Co. (St. Louis, MO). Timolol maleate, nadolol and atenolol were generously supplied by Banyu Pharmaceutical Co. (Tokyo), Dainippon Pharmaceutical Co. (Osaka) and I.C.I. Pharma Co. (Osaka), respectively. Ceramide (Type III from bovine brain sphingomyelin) and cholesteral-3-sulfate were obtained from Sigma Chemical Co. Palmitic acid and cholesterol were obtained from Tokyo Chemical Industry Co. (Tokyo) and Ishizu Pharmaceutical Co. (Osaka), respectively, and phosphatidylcholine (soybean lecithin) was the gift of Nihon Shoji Co. (Osaka). 1,6-Diphenyl-1,3,5-hexatriene (DPh), dansylhexadecylamine (DHA) and cholesterol anthracene-9-carboxylate (CAC) were obtained from Nacalai Tesque Co., Molecular Probes Inc. (USA) and Lambda Probes & Diagnostics Co. (Austria), respectively. Azone was generously supplied by Nelson-Sumisho Co. (Tokyo). DMSO and OA was obtained from Ishizu Pharmaceutical Co. and Nacalai Tesque Co., respectively. Other chemicals, obtained commercially, were of reagent grade. All these materials were used without further purification.

Preparation of Model Lipid Liposomes Multilamellar liposomes (MLV) of the model lipids were prepared by a hydration method. In brief, a mixture of ceramide (40%), cholesterol (25%), palmitic acid (25%) and cholesterol 3-sulfate (10%) was selected as the model lipids of the stratum corneum intercellular space.1-3 The lipid mixture was dissolved in chloroform-methanol (2:1 by volume) at the concentration of 1 mg/ml and 10 ml of the solution was transferred to a vacuum flask. The solvent was then removed under reduced pressure, and the resulting dried lipid film was hydrated with a 10 ml of pH 7.4 isotonic phosphate buffer containing each probe (DPh, DSHA or CAC) and each enhancer (Azone, OA or DMSO). A stock solution of 1 μM of each probe in tetrahydrofuran (THF) was prepared and stored at -5°C with protection from light. A small volume of each probe solution in THF was injected with rapid stirring into 2000, 1000 and 670 vol for DPh, DSHA and CAC, respectively, of isotonic phosphate buffer solution (pH 7.4) at room temperature. Each of the enhancers was then added to the solution at various concentrations. The solution was stirred overnight after which little or no odor of THF was detected, resulting in 0.5, 1.0 and 1.5 μM of...
DPH, DSHA and CAC, respectively. A thin layer of model lipid film was hydrated with this solution by vortexing at 80 °C for 5 min and MLV liposomes were obtained. The prepared liposomes were left for 2h and used for lipid fluidity measurements.

**Determination of Lipid Fluidity of Model Lipid Liposome Membrane**

Enhancer-induced changes in the lipid fluidity of model lipid liposome membranes were measured by the fluorescence polarization technique using DPH, DSHA and CAC as probes for hydrophobic lipid, polar head group and cholesterol regions, respectively. 

Fluorescence polarization was measured using a Hitachi MPF-4 fluorospectrophotometer equipped with polarizers and thermoregulated cells (at 37 °C). The excitation wavelengths were 360, 350 and 360 nm for DPH, DSHA and CAC, respectively, and the emission wavelengths were 430, 480 and 450 nm for DPH, DSHA and CAC, respectively. The degree of polarization \( P \) was calculated by the following equation:

\[
P = \frac{(I_{VV} - C_{I_{VH}})/(I_{VV} + C_{I_{VH}})}{(I_{VV} + C_{I_{VH}})/(I_{VV} - C_{I_{VH}})}
\]

where \( I \) is the fluorescence intensity, and subscripts \( V \) and \( H \) refer respectively to the vertical and horizontal orientations of the excitation (first) and emission (second) polarizers. \( C_{I} \) is a correction factor \( (C_{I} = I_{VH}/I_{VV}) \).

**Electron Microscopy**

Liposome suspension was placed on a thin copper specimen carrier plate and frozen in liquid nitrogen. Freeze-fracturing was carried out in an Eiko FD-2A apparatus. After etching treatment, the specimen was shadowed with platinum and coated with carbon. The replicas were obtained by dissolving liposomal materials with a hypochlorous acid and were then examined in a transmission electron microscope (H-800, Hitachi).

**Isolation of Rat Abdominal Skin**

Shaved abdominal skin without the adipose tissue was carefully excised from male Wistar rats (200-250 g) as described previously. 

**Drug Permeation Experiments**

Franz type diffusion cell having an available diffusion area of 3.14 cm² was employed. The receptor compartment was filled with 18 mL of isotonic phosphate buffer solution (pH 7.4). The tissue preparation was mounted in the cell with the stratum corneum facing upwards. Drug solutions were prepared with isotonic buffer solutions (pH 7.4) containing enhancers. The concentration of each enhancer was 0, 0.5, 1, 2, 5, 10% (w/v), 0, 0.2, 0.5, 1, 2, 5% (w/v) and 0, 20, 40, 60, 80% (w/v) for Azone, OA and DMSO, respectively. The drug concentration of pindolol was 2 mM because of its limited solubility; other drug solutions were made to be 5 mM. Two mL of each solution was applied to the donor compartment and the donor chamber was closed from the ambience. The diffusion cell was thermostated with a water jacket at 37 °C and the receptor compartment was stirred continuously at 600 rpm with a magnetic stirrer. At appropriate times, an aliquot of the receiver fluid (0.2 mL) was withdrawn and the same volume of fresh buffer solution was applied to the receptor compartment. Sample solution was filtered through a 0.45 μm pore-size filter (Nihon Millipore Kogyo, Yonezawa) for the analysis.

**Measurement of Drug Solubility**

An excess amount of each drug was added to an aliquot of each vehicle solution containing enhancer at each concentration and was agitated at 37 °C for 12 h in a shaking bath. The resulting suspension was centrifuged and the supernatant was filtered through a 0.45 μm pore-size filter. The diluted filtrate was applied to HPLC to determine the drug concentration.

**Analytical Methods**

Drug concentration was determined by HPLC. An HPLC pump (LC-5A, Shimadzu, Kyoto) was equipped with a UV detector (SPD-2A, Shimadzu) or a fluorescence detector (RF-530, Shimadzu). The system was used in a reversed phase with an Inertsil ODS column (4.6 i.d. x 150 mm, GL Sciences Inc., Tokyo). A mixture of methanol and 10% acetic acid was used as the mobile phase at a flow rate of 1.0 mL/min. The analytical conditions are listed in Table I. Cumulative amounts of the drug penetrated and the apparent permeability coefficient were calculated by the method reported previously. 

The maximum flux (\( J_{\text{max}} \)) has been used to describe the intrinsic permeability of the skin to a specific penetrant. Since the thermodynamic activity can be approximated by the ratio of the drug concentration to its saturated solubility in the vehicle, \( J_{\text{max}} \) can be calculated by the earlier described method.

### Results and Discussion

**Effect of Enhancer on the Fluidity of Model Lipid Liposome Membrane**

Figure 1 shows a change in fluorescence polarization of DPH, which is a probe for hydrophobic lipid chains, in the model lipid liposomes as a function of enhancer concentration (mol% ratio to total lipid). A decrease of polarization (\( P \) value) represents an increase in lipid fluidity. As shown in Fig. 1, DMSO had no effect on the lipid fluidity even at high enhancer concentrations. The lipid fluidity was markedly increased, however, by certain added concentrations of Azone and OA. Figure 2 shows results of the change in polarization of DSHA, representing lipid fluidity of the polar head group. DMSO also had no effect on the fluidity of the polar head group. But the lipid fluidity was remarkably increased by the addition of Azone and OA up to 20 mol% where they reached a plateau. As

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**Table I.** HPLC Conditions for Drug Concentration Measurements

<table>
<thead>
<tr>
<th>Drug</th>
<th>UV wavelength (nm)</th>
<th>Fluorescence wavelength (Ex nm, Em nm)</th>
<th>Mobile phase MeOH:10% AcOH (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propranolol</td>
<td>290</td>
<td>295, 340</td>
<td>45:55</td>
</tr>
<tr>
<td>Metoprolol</td>
<td>273</td>
<td>272, 308</td>
<td>30:70</td>
</tr>
<tr>
<td>Timolol</td>
<td>290</td>
<td>250, 302</td>
<td>30:70</td>
</tr>
<tr>
<td>Pindolol</td>
<td>269</td>
<td>269, 303</td>
<td>20:80</td>
</tr>
<tr>
<td>Nadolol</td>
<td>274</td>
<td>270, 305</td>
<td>20:80</td>
</tr>
</tbody>
</table>

*By volume.*

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**Fig. 1. Effect of Each Enhancer on Fluorescence Polarization of DPH Using Model Lipid Liposome**

Each value of \( P \) represents the mean of 3 experiments. --- DA; -- DMSO; - Azone.

**Fig. 2. Effect of Each Enhancer on Fluorescence Polarization of DSHA Using Model Lipid Liposome**

Each value of \( P \) represents the mean of 3 experiments. --- DA; -- DMSO; - Azone.
these enhancer molecules are lipophilic substances, the enhancer molecules in the aqueous exterior solution might partly associate to form molecular aggregates, and through hydrophobic interaction might partially concentrate around the polar head group of the bilayer surface of the liposome. The enhancer concentration might therefore be relatively high around this group, which might result in a sensitive fluidity change of polar head group. Further, because of the balance with the force required to retain the bilayer system of the liposome, the increment of lipid fluidity in the polar head group might not be as large as that in the hydrophobic carbon chain. The change in polarization of CAC, representing fluidity of the cholesterol chain, is shown in Fig. 3. Similar results were seen in the cholesterol chain. The membrane fluidity was thus apparently remarkably increased in each part of the membrane by the addition of Azone and OA, while DMSO had little effect on the fluidity. The enhancing mechanism of DMSO is not a change in intercellular lipid fluidity, but its direct effect on corneocytes. These results may suggest these view.

Since Azone has a relatively high affinity to the model lipids, lipid fluidity may be critical in maintaining a multi-layer structure at high Azone concentrations. To confirm the integrity of the liposome containing a high Azone concentration, model lipid liposome containing 100 mol% Azone was observed by electron microscope observation and revealed a multi-layer structure of the liposome (Fig. 4). The size of the liposome shown in Fig. 4 was recognized to be the same as the control liposome containing no enhancer (1–3 μm). Integrity of the liposome containing 100 mol% Azone was thus confirmed. The liposome containing 100 mol% OA also showed no change in size.

**In Vitro Enhancement Effect on Rat Skin Permeation**

Figure 5 shows the change in permeability coefficients ($K_p$) of six β-blockers of different lipophilicities through rat abdominal skin following addition of each enhancer. The $K_p$ value for each drug was remarkably increased by Azone, and maximum effect was obtained at a concentration range of 0.5—1.0% (w/v) for all drugs. A similar result was possible with OA, but the effect was not as large as that with Azone. The enhancement effect of DMSO was seen at high concentrations of over 60% (w/v), but was barely observable at low concentrations. Thus, no enhancement of percutaneous absorption can be expected when DMSO is used as an enhancer. The drug maximum flux ($J_{max}$) was calculated to correct the drug activity changes in the vehicle.22 Figure 6 shows changes in the enhancement index of $J_{max}$ ($J_{max}$ with enhancer/$J_{max}$ without enhancer) with the

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**Fig. 3.** Effect of Each Enhancer on Fluorescence Polarization of CAC Using Model Lipid Liposome
Each value of $P$ represents the mean of 3 experiments. — □ —, OA; — ● —, DMSO; — ■ —, Azone.

**Fig. 4.** Freeze-Fracture Micrograph of Model Lipid Liposomes Formed from a Mixture of 40% Ceramide, 25% Cholesterol, 25% Palmitic Acid and 10% Cholesterol 3-Sulfate Containing 100 mol% Azone (× 40000)

**Fig. 5.** Enhancement Effect of Each Enhancer, Azone (a), OA (b), DMSO (c), on Drug Permeability Coefficient ($K_p$) in Rat Skin
Each value of $K_p$ represents the mean of 3 experiments. ■ propanol; □ metoprolol; ● timolol; ○ pindolol; ○ nadolol; ■ atenolol.
addition of each enhancer, and indicate that the order of enhancement ability is Azone > OA > DMSO. The order of drug lipophilicity is propranolol > metoprolol > timolol > pindolol > nadolol > atenolol. Therefore, the effects of Azone and OA were more pronounced in hydrophilic drugs than in lipophilic drugs. The increment of lipid fluidity and resulting enhanced water permeability might be the reason for this phenomenon.

Information on the enhancement mechanism was sought in drug diffusion constants (D) in the rat skin. Figure 7 shows the relationship between the enhancement index of D and the enhancer concentration. The D value of six drugs were increased remarkably by the addition of Azone, while with OA the increase was not as large. Little effect of DMSO was observed unless high concentrations were employed. These results were consistent with those of lipid fluidity measurements. In Fig. 7a and b, the enhancement indexes of D value for pindolol at high enhancer concentrations were far larger than those expected from the lipophilicity, indicating that there may be an interaction between the drug and the enhancer molecules accumulated in the stratum corneum layer. To resolve this phenomenon requires further investigation such as measurements of enhancer concentration in the stratum corneum.

Several suggestions have been advanced regarding the mode of action of DMSO: (1) the extraction of stratum corneum lipids, lipoproteins and nucleoproteins; (2) displacement of bound water and loosening of the polymeric structure within the corneocytes; and (3) osmotically inducing delamination of the stratum corneum. Scheuplein suggested that the intense hygroscopicity causes the displacement of normally bound water to a more structured and loosely bound form. This would enhance the passage of water and other molecules. The mechanism of action whereby Azone enhances skin permeability remains unresolved. There are indications that an ion-pairing type mechanism may be operative for some permeants, and this will be discussed in more detail later. There do not appear to be any gross morphological changes within the horny layer following treatment with low concentrations of the enhancer. The alteration of stratum corneum components can be detected by differential scanning calorimetry and Fourier transform infrared spectroscopy (FTIR). The former has proved useful in detecting changes in lipid transitions in enhancer-treated skin, suggesting that Azone probably acts by disrupting horny layer lipid structure. Fatty acids such as OA possibly cause an even greater increase in stratum corneum lipid fluidity. The mode
of action undoubtedly targets the lipids of the stratum corneum.\textsuperscript{25-37}

In this study, three kinds of enhancers with different modes of action were evaluated from the standpoint of their lipid fluidizing effect using the model lipid liposome, and the \textit{in vitro} effects of the enhancers on drug permeations through rat skin were measured. From the results it was clear that the stronger the effect of the enhancer on the model lipid fluidity increment, the larger was the \textit{D} value of a drug in the skin.

\textbf{Conclusions}

To investigate the usefulness of the model lipid liposome as a system for evaluating the enhancing ability of various penetration enhancers, the lipid fluidity changes of the model lipid liposome membrane consisting of ceramide, cholesterol, palmitic acid and cholesterol sulfate, by Azone, OA or DMSO, were measured by the fluorescence polarization method; \textit{in vitro} drug permeation studies using rat skin were also performed. The result obtained confirmed that the system was appropriate for making such evaluations.

\textbf{References}

2) P. M. Elias, \textit{Arch. Dermatol.}, 270, 95 (1981).