Promoting Mechanism of Menthol Derivative, 1-O-Ethyl-3-butylycyclohexanol, on the Percutaneous Absorption of Ketoprofen

Chao Jie Li, a Kimio Higashiyama, b Yoshihiro Yoshimura, c Tsuneji Nagai, e Kozo Takayama, e and Yasuko Obata b d

Department of Pharmaceutics, a Department of Organic Chemistry, b and Department of Analytical Chemistry, c Hoshi University, 2-4-41 Ebara, Shinagawa-ku, Tokyo 142-8501, Japan. Received March 27, 2001; accepted June 13, 2001

Menthol derivatives were synthesized and evaluated for their promoting activity on the percutaneous absorption of ketoprofen and skin irritation in vivo, choosing O-ethylmenthol (MET) as the mother compound. The compound having a C-3 positioned n-butyl group (1-O-ethyl-3-n-butylycyclohexanol, OEBC) indicated the most promoting activity and caused relatively little skin irritation. In order to understand enhancement mechanism of OEBC, an in vitro permeation study of ketoprofen was performed. The time course of the cumulative amounts of drug permeated through the rat skin exhibited a linear relation after an initial lag time. This was analyzed in membrane diffusion model and the diffusion and partition parameters of ketoprofen were estimated. Both parameters were remarkably enhanced when a hydrogel containing a small quantity of OEBC (0.5%) was applied. Furthermore, to clarify the site of action of OEBC, we also investigated in vitro permeation study of ketoprofen employing different states of skin, reversed skin and stratum corneum stripped skin. When OEBC was added to the hydrogels which were applied to the reversed and stripped skins, almost no changes of the flux were observed compared with the control (without OEBC). These results suggested that the site of action of OEBC was stratum corneum. Morphological changes of the stratum corneum surface were microscopically observed with 0—2% OEBC. The spaces between the stratum corneum cells treated with 0.5—2% OEBC became extended and the shape of each cell became clear. This may suggest that the site of action of OEBC was the intercellular of stratum corneum. Furthermore, an electron spin resonance study was performed to investigate the effect of OEBC on the intercellular lipid bilayer fluidity of the stratum corneum and the rotational correlation times were calculated. 2,2,6,6-Tetramethylpiperidine-1-oxyl (TEMPO) and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL) were used as the spin label. In use of OEBC, the fluidity of TEMPO labeled the stratum corneum lipid increased as the addition of OEBC. The results suggested that OEBC promote the penetration of drugs by enhancing fluidity of the local lipid bilayers around TEMPO.

Key words: menthol derivative; 1-O-ethyl-3-butylycyclohexanol; percutaneous absorption; electron spin resonance; scanning electron microscope

Transdermal drug delivery is an effective method to deliver drugs into systemic circulation for the desired therapeutic effect. However, the barrierability of the stratum corneum to foreign substances must be overcome. To overcome this barrierability, an absorption enhancer was usually added to the transdermal formulation. It has been found that cyclic monoterpenes such as d-limonene and l-menthol remarkably enhanced the skin permeability of several kinds of drugs (Okabe et al., 1989; Obata et al., 1990; Takayama et al., 1991; Ohara et al., 1994). 1-4 O-Alkylmenthol and O-acyl-menthol derivatives were also synthesized and investigated their ability to enhance percutaneous absorption of ketoprofen from alcoholic hydrogels in rats in vivo and in vitro (Negishi et al., 1995; Nakamura et al., 1996). 1-6 Among those compound, O-ethylmenthol (MET) showed the greatest efficiency at very low concentrations and caused relatively little skin irritation.

Recently, we synthesized menthol derivatives which have mono- or di-substitute groups and an O-ethyl group (Obata et al., 2000). 1 Their enhancement activity and the skin irritation for percutaneous absorption of ketoprofen was evaluated in rats in vivo. In vivo study showed that the promoting activity of 1-O-ethyl-3-butylycyclohexanol (OEBC) was approximately 2-fold compared with that of MET. The skin irritation, however, was almost same as MET. Therefore, OEBC was considered a powerful and new skin penetration enhancer.

In this study, we focused on the promoting mechanism of OEBC. The skin permeation study of ketoprofen was performed using rat skin in vitro and the diffusion and partition parameters of ketoprofen were determined. Furthermore, to clarify the site of action of OEBC, we also investigated in vitro permeation study employed different state of skin, i.e. reversed or stripped skin. Morphological changes of the skin surface of hairless rat were observed with a scanning electron microscope (SEM) after application of hydrogels containing OEBC. Furthermore, the influence of OEBC on the intercellular lipid bilayer fluidity was determined using electron spin resonance (ESR) spectroscopy.

MATERIALS AND METHODS

Materials The chemical structures of MET and OEBC were shown in Fig. 1. OEBC was synthesized by the method described by Y. Obata et al. (2000), 1 and was characterized by elemental analysis, nuclear magnetic resonance (NMR) spectroscopy (Jeol GSX 270F, Tokyo, Japan) and gas chro-

Fig. 1. The Chemical Structures of Absorption Enhancers
a) O-Ethylmenthol (MET), b) 1-O-ethyl-3-butylycyclohexanol (OEBC).
matography (GC) (Shimadzu GC-7A, Kyoto, Japan). The purity was over 99%. Ketoprofen was purchased from Sigma Co. (St. Louis, MO, U.S.A.). Carboxyvinyl polymer (HIVIS-WAKO 105) was generously supplied by Wako (Osaka, Japan). 2,2,6,6-Tetramethylpiperidine-1-oxyl (TEMPO) and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All other chemicals used were of reagent grade.

Preparation of Hydrogel The formulae of the ketoprofen hydrogels used in this study are listed in Table 1. The hydrogels were prepared as follows: carboxyvinyl polymer and triethanolamine were dissolved in distilled water. Separately, ketoprofen and each of the enhancers were dissolved in ethanol. Both solutions were mixed and the resulting hydrogel was stored at room temperature for 24 h under air-tight conditions prior to use.

In Vitro Skin Permeation Study Full-thickness abdominal skin was excised from male Wistar rats weighing 180—200 g, whose hair had been previously removed with an electric clipper. The excised skin was used as a permeation membrane. Franz diffusion cell having an available diffusion area of 1.77 cm² was employed. The receiver side was filled with 16 ml of phosphate buffer solution (pH 7.2) and the donor side was filled with test hydrogel (1.0 g) under occlusive conditions. Franz cell was thermoregulated at 37°C and the receiver side was stirred with a magnetic stirrer. At appropriate times, an aliquot of the receiver fluid (50 µl) was withdrawn and the same volume of fresh buffer solution was supplied to the receiver side. Each aliquot was mixed with methanol (500 µl) containing an appropriate amount of p-hydroxybenzoic acid n-hexyl ester as an internal standard. The sample was filtered through a disposable filter unit (Ekikuro-Disc 3CR, Gelman Science Japan, Tokyo, Japan). The concentration of ketoprofen in the filtrate was analyzed using a high-performance liquid chromatography (HPLC) system (Shimadzu, LC-10AS) equipped with a variable wavelength ultraviolet monitor (Shimadzu, SPD-6A). The column was a YMC-Pack A-302 S-5 120A ODS (4.6x150 mm; Yamamura Chemical Laboratories). The flow rate was 1.0 ml/min and elution was carried out at room temperature with a mobile phase consisting of 0.0570/0 aqueous phosphoric acid-methanol (35:65). The column effluent was monitored at 254 nm.

The permeation study with "reversed skin" was performed by the spin label (Keith et al., 1970; Marsh, 1981). For a pretreatment, the prepared stratum corneum were soaked in the 40% ethanol solution alone was performed as a control. After drying, the pretreated stratum corneum was treated by the procedure described above to be labeled with TEMPO or TEMPOL.

ESR Spectra Measurement Preparation of Stratum Corneum: The stratum corneum of the hair-removed abdominal skin of hairless rats was prepared as follows: The skin was placed, stratum corneum side up, on filter paper and floated on 0.5% trypsin (type II, Sigma) in a phosphate buffered saline (PBS, pH 7.4) for 24 h at 37°C. After incubation, the stratum corneum sheets was separated from the dermis by mild agitation of its sheets. Samples were dried for 12 h at room temperature over silica gel.

Spin-Labeling Procedures and Pretreatment of Stratum Corneum with OEBC: TEMPO and TEMPOL were selected as spin-labeling agents. A slice of the dried stratum corneum sheet (4.1x0.7 cm) were incubated in the TEMPO or TEMPOL buffer solution (pH 7.4, 10 µg/ml) for 2 h at 37°C.

For a pretreatment, the prepared stratum corneum were soaked in the 40% ethanol solution containing 0.5% of OEBC for 8 h at room temperature. Similar treatment with 40% ethanol solution alone was performed as a control. After drying, the pretreated stratum corneum was labeled with TEMPO or TEMPOL.

ESR Spectral Measurements: Spectral measurements were performed with an ESR spectrometer (Jeol JES-REX). X-band first derivative absorption spectra were obtained with a microwave power output of 8 mW; center field, 332 mT; time constant, 0.3x0.1 s; sweep time, 2.0 m; modulation, 0.63x0.1 mT at a frequency of 100 kHz; and total sweep width, 5.0 mT. The stratum corneum previously labeled with TEMPO or TEMPOL, were mounted on the flat surface of a quartz cell and located in the center of a TM cavity. Spectra were collected by a ESR data format COMPRES. Each sample was scanned three times and the ESR parameters from each spectrum were averaged to give a single estimate for that sample.

An empirical parameter, the rotational correlation time (τ), can be used to give a measure of motion in the region probed by the spin label (Keith et al., 1970; Marsh, 1981). The equation is:

\[ r = 6.5 \times 10^{-10} \frac{W_y}{(h_y/\ell_1)^{1/2}} \]  

where \( W_y \) is the line width in G; \( h_y \) and \( \ell_1 \) are the heights of the mid- and high-field lines, respectively, on a first-derivative absorption spectrum.

Statistical Analysis For statistical evaluation of results, the one-way analysis of variance (ANOVA) were employed. A p-value smaller than 0.05 was considered as significant.

<table>
<thead>
<tr>
<th>Table 1. Formulae of Ketoprofen Hydrogel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ketoprofen</td>
</tr>
<tr>
<td>Carboxyvinylpolymer</td>
</tr>
<tr>
<td>Triethanolamine</td>
</tr>
<tr>
<td>Ethanol</td>
</tr>
<tr>
<td>Enhancers</td>
</tr>
<tr>
<td>Water</td>
</tr>
</tbody>
</table>
RESULTS AND DISCUSSION

The Effects of OEBC on the Permeation of Ketoprofen through Rat Skin

Figure 2 shows the permeation profiles of ketoprofen in hydrogels containing OEBC through rat skin in vitro. The cumulative amount of ketoprofen linearly increased with an increase of time after certain lag time. With the addition of OEBC, the permeation of ketoprofen was remarkably enhanced compared with the control (without OEBC). The flux was estimated from the slope of the permeation profiles. As shown in Fig. 3, when a small amount of OEBC (0.125—0.5%) was added to the hydrogels, the flux of ketoprofen was remarkably increased compared with the control (without OEBC). While further increases in the amounts of OEBC (1—2%), no increase in the flux was observed. From these results, it was suggested that the maximum enhancing effect was obtained with 0.5% OEBC. Therefore, it was considered that 0.5% was sufficient enough to exhibit maximum enhancing activity.

The profiles of ketoprofen permeation through the skin, as shown in Fig. 2, were analyzed by a method described by Okamoto et al. (1988), based on the following diffusion model.

\[ D' = \frac{D}{L^2} \]

(2)

\[ K' = KL \]

(3)

\[ Q_t = AK'C_o \left[ \frac{1}{6} \sum_{n=0}^{\infty} \frac{(-1)^n}{n^2} \times \exp\left(-D'\pi^2 n^2 t\right) \right] \]

(4)

\[ D \] is the diffusion constant, \( L \) is the thickness of the membrane, \( K \) is the partition coefficient of the penetrant between the membrane and the donor phase, \( Q_t \) is the cumulative amount of penetrant in the receptor fluid at time \( t \), \( A \) is the area of application, and \( C_o \) is the concentration of the donor phase. The diffusion parameter, \( D' \), and the partition parameter, \( K' \), were simultaneously estimated by a curve-fitting technique employing a computer program (MULTI) (Yamashita et al., 1981). Results were shown in Fig. 4. The \( D' \) value of ketoprofen was remarkably increased with increasing OEBC concentrations (0.5—2%) in the hydrogels. The \( D' \) of 1% OEBC showed the highest value. The \( K' \) value was significantly enhanced when OEBC was added to the hydrogels. This may suggest that a small amount of OEBC (0.125—0.25%) contributed only increase in lipophilicity of skin surface, however, didn’t change the dense barrier structure of stratum corneum. To overcome this barrier, at least 0.5—1% OEBC were required in the hydrogel. These results indicated that the partitioning of ketoprofen from the hydrogel to the skin is improved by the addition of a small amount of OEBC (0.5%), and the diffusivity of the drug is also enhanced.

The Effect of Different State of Skin on the Permeation of Ketoprofen

To confirm the site of action of OEBC, different state of skin was used for the in vitro study. Figure 5 showed the results of permeation of the reversed or stripped
In the Franz cell. When the hydrogel containing 0.5% OEBC was applied, little changes of the flux were observed compared with the control (without OEBC). The promoting effect of OEBC was disappeared in either "reversed" or "stripped" skin, suggesting that the site of action of OEBC is in the stratum corneum.

**The Effects of OEBC on the Morphology of the Skin**

![Effect of OEBC on the Flux of Ketoprofen through the Various State of Excised Rat Skin](image)

**Surface** Morphological changes in the skin surface treated with hydrogels containing OEBC were examined by a scanning electron microscope (SEM). In this study, the abdominal skin excised from hairless rats was used because the skin surface of Wistar rats, which had been used in past absorption studies, is covered with rather thick hair. Figure 6 showed typical examples of microscopic photographs of the skin surface treated with hydrogels containing OEBC (0.125—2%) for 8 h. The intact skin surface (without OEBC) showed rough and irregular morphology (Fig. 6(a)). However, the roughness of the skin surface gradually decreased with an increase in the OEBC concentration. In particular, remarkable changes in the skin surface were observed with 0.5—2% OEBC. The intercellular space was enlarged and the shape of each cell became clear (Figs. 6(b)—(f)). The intercellular region of the stratum corneum consists of major bilayer-forming lipids such as ceramides, cholesterol, free fatty acids and cholesterol sulfate (Abraham and Downing, 1991; Kim et al., 1993; Ongpipattanakul et al., 1994; Michel et al., 1995). OEBC would be distributed to the intercellular lipid region and may change the dense barrier structure of the stratum corneum. It was suggested that the site of action of OEBC was the intercellular lipid bilayers of stratum

![Effect of OEBC](image)
The Effects of OEBC on Intercellular Lipid Fluidity

To clarify the mechanism of action of OEBC, we measured ESR spectra of TEMPO and TEMPOL labeled stratum corneum treated with OEBC. When the spin probes move freely, sharp triplet signals can be seen. However, when the mobility of the spin probes is restricted by the interaction with other molecules, the ESR spectrum is split. Figure 7(a) shows the typical ESR spectrum observed with TEMPO labeled stratum corneum, which was treated with 0.5% OEBC. The rotational correlation time (τ) was estimated from the spectrum shown in Fig. 7(a). As a result, the τ value was estimated to be $1.138 \times 10^{-11}$ s (Fig. 7(a)), suggesting microviscosity of the stratum corneum lipid was significantly lowered by the treatment of OEBC, compared with the control (without OEBC $2.454 \times 10^{-11}$ s). On the contrary, the τ value ($4.440 \times 10^{-12}$ s) (Fig. 7(b)) for TEMPOL were almost equal to the control (without OEBC $4.547 \times 10^{-12}$ s), suggesting OEBC did not affect the fluidity of the hydrophilic domain in the lipid bilayer. In conclusion, OEBC was located in the intercellular lipid of stratum corneum and bring about an increase in the fluidity of the local lipid bilayer. Therefore, increase in the fluidity of the intercellular lipid bilayer by OEBC seems to permit much easier drug diffusion through the skin, which will promote the penetration of drugs.

Acknowledgements

This study was supported by the Ministry of Education, Science, Sports and Culture of Japan. The animal experiments were conducted in accordance with the Guide for Care and Use of Laboratory Animals adopted by the Committee on Care and Use of Laboratory Animals of Hoshi University which is accredited by the Ministry of Education, Science, Sports and Culture, Japan.

REFERENCES