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Promoting effect of *O*-ethylmenthol on the percutaneous absorption of ketoprofen

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Abstract

The promoting effect of *O*-ethylmenthol (MET) on the percutaneous absorption of ketoprofen from alcoholic hydrogels was evaluated in rats in vitro and in vivo. Further, the anti-inflammatory action of ketoprofen hydrogels was evaluated with a rat paw edema test. The time course of the cumulative amounts of drug permeated through the rat skin in vitro exhibited a linear relation after an initial time lag. This was analyzed in a 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 MET (0.5%) was applied. However, at least 2% menthol was required to obtain the same activity as 0.25% MET. A pharmacokinetic model, which was derived on the assumption of a constant penetration rate (R_p) after a lag time, was employed to evaluate in vivo percutaneous absorption of ketoprofen from hydrogels containing MET. Further, the area under the plasma concentration–time curve ($AUC_{0-8\,h}$) was estimated. Similarly to the results observed in vitro, R_p and $AUC_{0-8\,h}$ values were increased significantly by the administration of hydrogels containing a small amount of MET (0.25–0.5%). In order to obtain the significant inhibitory action of ketoprofen on the rat paw edema induced by carrageenan, at least 1% menthol was required in the hydrogel formulation. On the other hand, a small amount of MET (0.25–0.5%) was enough to bring about significant inhibitory action of ketoprofen. Distinguishable changes of the skin surface were microscopically observed with 0.5–2% MET, i.e. the spaces between the stratum corneum cells became extended and the shape of each cell became clear, whereas the morphological changes caused by menthol were relatively weak. Both MET and menthol may change the dense barrier structure of the stratum corneum of skin; however, the efficiency of MET is significantly greater than that of menthol. Copyright © 1996 Elsevier Science B.V.

Keywords: Absorption enhancer; Ketoprofen; Menthol; *O*-Ethylmenthol; Percutaneous absorption

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1. Introduction

Development of a transdermal therapeutic system will depend on the ability of drugs to penetrate the stratum corneum and deliver sufficient concentrations of the drug into systemic circulation for the desired therapeutic effect. To achieve this, an absorption enhancer usually needs to be included in the transdermal formulation. Many studies have discussed percutaneous absorption enhancers and mechanisms of their enhancing activity (Barry, 1987; Okamoto et al., 1988; Kadir and Barry, 1991). We have 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). Recently, we synthesized *O*-alkylmenthol and *O*-acylmenthol derivatives, and investigated their ability to enhance percutaneous absorption of ketoprofen from alcoholic hydrogels in rats in vivo (Negishi et al., 1995). The skin irritancy of these compounds was also evaluated based on histopathological findings (Negishi et al., 1995). *O*-ethylmenthol (MET, Fig. 1) was the most promising compound, with the greatest promoting action and relatively low skin irritancy. In this study, we focused on how MET promotes absorption. The effect of MET on the percutaneous absorption of ketoprofen was investigated in rats in vitro and in vivo, and compared with the effects of menthol. The anti-inflammatory activity of ketoprofen hydrogels containing MET or menthol was also evaluated employing a rat paw edema test. Furthermore, morphological changes of the skin surface of hairless rat were observed with a scanning electron microscope after application of hydrogels containing MET or menthol.

2. Materials and method

2.1. Materials

l-Menthol of extra pure reagent grade, was purchased from Tokyo Kasei Industrial Company (Tokyo, Japan). MET was synthesized by the method described by Leffler and Calkins (1955),

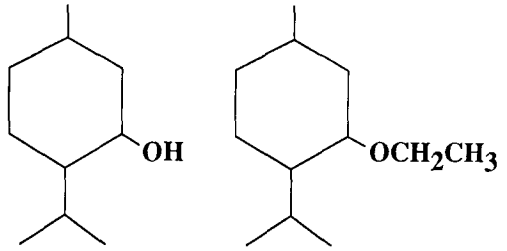

Fig. 1. The chemical structures of menthol and MET.

and was characterized by elemental analysis, nuclear magnetic resonance (NMR) spectroscopy (Jeol GSX 270F, Tokyo, Japan) and gas chromatography (GC) (Shimadzu GC-7A, Kyoto, Japan). The purity of this compound was over 99%. Ketoprofen was purchased from Sigma (St. Louis, MO, USA). Carboxyvinyl polymer, marketed as Hiviswako 105, was generously supplied by Wako Pure Chemical Industries (Osaka, Japan). λ-Carrageenan was purchased from Zushi Kagaku Laboratory (Kanagawa, Japan). The other chemicals used were of reagent grade.

2.2. Preparation of hydrogels

The formulae of ketoprofen hydrogels are listed in Table 1. The hydrogels were prepared as follows: ketoprofen was dissolved in ethanol containing menthol or MET. Separately, carboxyvinyl polymer and triethanolamine were dissolved in distilled water. Both components were then mixed well and the resulting hydrogel was stored at room temperature for 24 h under airtight conditions prior to use.

Table 1
Formulae for the ketoprofen hydrogels

Ketoprofen (g)	3.0
Carboxyvinylpolymer (g)	1.5
Triethanolamine (g)	2.0
Ethanol (g)	40.0
Enhancers (g)	0–2.0
Water (g)	100.0 (ad)

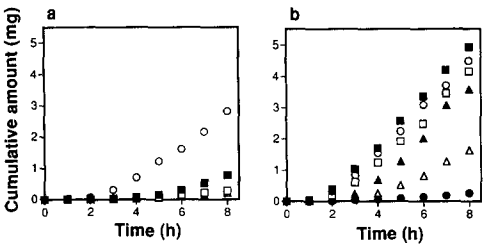

Fig. 2. Effect of menthol (a) or MET (b) on the permeation of ketoprofen from hydrogels in rat skin. Each point is the mean of three determinations. Concentrations of enhancer: (●) 0%; (△) 0.125%; (▲) 0.25%; (□) 0.5%; (■) 1%; (○) 2%.

2.3. 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 for the in vitro study. The vertical diffusion cell having an available diffusion area of 1.77 cm^2 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 (1.5 ml) under occlusive conditions. The vertical 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 (20 μg) was withdrawn and the same volume of fresh buffer solution was supplied to the receiver side. Each sample was mixed with methanol (200 μg) 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-6A, Kyoto, Japan) equipped with a variable wavelength ultraviolet monitor (Shimadzu, SPD-6A). The flow rate was 1 ml/min and elution was carried out at room temperature. The other analytical conditions were as follows: column, YMC-Pack A-302 S-5 120A ODS, 150 × 4.6 mm i.d. (YMC, Tokyo, Japan); ultraviolet detection, 254 nm; mobile phase, 0.057% phosphoric acid/methanol (35:65).

2.4. Percutaneous absorption study

Male Wistar rats weighing 180–200 g were anesthetized with a carbamic acid ethyl ester solution (25%, 4 ml/kg intraperitoneally (i.p.)) and secured on their backs. The abdominal hair was gently removed with an electric clipper. A glass cell with a 16-mm inner diameter and 10 mm in height, was attached on the shaved abdominal skin with a cyanoacrylate type adhesive (Aron Alpha A, Sankyo, Tokyo, Japan) and filled with the test hydrogel (1 ml) under occlusive conditions. Blood samples (300 μl) were taken via the jugular vein 1, 2, 4, 6 and 8 h after application. Each blood sample was centrifuged for 1 min and the plasma sample (100 μg) was thoroughly mixed with methanol (300 μg) containing an appropriate amount of *p*-hydroxybenzoic acid *n*-hexyl ester as an internal standard. The mixture was centrifuged again for 1 min to precipitate the denatured proteins. The supernatant solution was filtered using a disposable filter unit. The concentration of ketoprofen in the filtrate was analyzed as in the in vitro permeation study.

2.5. Anti-inflammatory activity

The anti-inflammatory activity of ketoprofen hydrogels containing menthol or MET was evaluated with a rat paw edema test. In the same manner as described in the percutaneous absorption study, the hydrogel (1 ml) was applied to the shaved abdominal skin of male Wistar rats weighing 180–200 g. Just before administration of hydrogels, 1% carrageenan–saline solution (0. 1 ml) was injected into each hindpaw of rats. The thickness of paw edema induced by carrageenan was measured by a slide caliper during 8 h after the irritant treatment. Results were statistically analyzed by means of Dunnet's multiple comparison test.

2.6. Morphological observation

Male WBN hairless rats weighing 180–200 g were anesthetized with a carbamic acid ethyl ester solution (25%, 4 ml/kg i.p.) and secured on their backs. A glass cell with a 12-mm inner diameter

Table 2
Effect of enhancer concentrations on the diffusion parameter (D') and partition parameters (K') of ketoprofen

Enhancer	Concentration (%)	$D' \times 10^2$ (h^{-1})	$K' \times 10$ (cm)
Menthol	0.125	3.44 ± 0.40	0.335 ± 0.195
	0.25	3.78 ± 0.71	0.374 ± 0.065
	0.5	3.45 ± 0.48	0.476 ± 0.175
	1	2.69 ± 0.18	1.31 ± 0.08
	2	4.89 ± 1.19	2.51 ± 0.82
MET	0.125	3.75 ± 0.54	1.86 ± 0.87
	0.25	3.77 ± 0.71	2.54 ± 0.83
	0.5	5.79 ± 0.81	2.61 ± 0.10
	1	8.66 ± 0.12	1.83 ± 0.43
	2	8.04 ± 0.98	1.82 ± 0.43
Control (without enhancer)		3.41 ± 0.55	0.440 ± 0.120

Each value represents the mean ± S.D. of three determinations.

and 15 mm in height, was attached on the shaved abdominal skin with a cyanoacrylate-type adhesive (Aron Alpha A, Sankyo, Tokyo, Japan) and filled with the test hydrogel (0.7 ml) under occlusive conditions. The skin was excised after 8 h exposure to the hydrogel, and the skin surface was washed with phosphate-buffered saline (PBS) (pH 7.3) and prefixed with a 2% glutaraldehyde buffer solution for 2 h. After being soaked in a 7.5% saccharose buffer solution (pH 7.3) for 24 h, the skin was fixed again with a 1% osmic acid buffer solution (pH 7.3) for 2 h. The skin was dehydrated in a graded ethanol series (60–100%) and then ethanol was replaced with *t*-butanol. After lyophilization (ES-2030, Hitachi, Tokyo, Japan), the skin surface was coated with gold (Jeol, Ionsputter JFC-1100, Tokyo, Japan) and examined under the scanning electron microscope (Jeol, JSM-T200, Tokyo, Japan).

3. Results and discussion

3.1. The effects of MET on the permeation of ketoprofen through rat skin

Fig. 2 shows the permeation profiles of ketoprofen in hydrogels containing MET or menthol through rat skin in vitro. When a small quantity of MET (0.25–0.5%) was added to the hydrogels, the permeation of ketoprofen increased remarkably, compared with the control (without an enhancer), while further increases in the amounts of MET (1–2%) only resulted in limited increases in permeation. Little change in permeation was observed when small amounts of menthol were used ($< 1\%$), and at least 2% menthol was required to obtain a promoting efficiency comparable with 0.25% MET. To find out how MET promotes permeation, the profiles of ketoprofen permeation through the skin, shown in Fig. 2, were analyzed by a method described by Okamoto et al. (1988), based on the following diffusion model.

$$Q_t = AK'C_0\left[D't - \frac{1}{6} - \frac{2}{\pi^2}\sum_1^{\infty}\frac{(-1)^n}{n^2}\exp(-D'n^2\pi^2t)\right] \quad (1)$$

$$D' = D/L^2 \quad (2)$$

$$K' = KL \quad (3)$$

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_0 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) (Yamaoka et al.,

Table 3
Pharmacokinetic parameters of the percutaneous absorption of ketoprofen in rats in vivo

Enhancer	Concentration (%)	$R_p \times 10^{-1}$ (μg/h)	$t_L \times 10$ (h)	AUC_{0-8h} (μg·h/ml)
Menthol	0.125	1.37 ± 0.35	10.6 ± 0.0	2.10 ± 0.47
	0.25	1.64 ± 0.34	10.6 ± 0.0	2.35 ± 0.42
	0.5	2.40 ± 0.21	10.6 ± 0.1	3.32 ± 0.09
	1	5.80 ± 0.56	10.4 ± 0.1	8.30 ± 0.60
	2	75.3 ± 2.12	0.618 ± 0.101	137 ± 6
MET	0.125	3.14 ± 0.97	10.4 ± 0.0	4.35 ± 1.41
	0.25	31.5 ± 9.8	10.1 ± 0.2	42.7 ± 13.1
	0.5	87.3 ± 25.5	0.678 ± 0.054	161 ± 57
	1	94.2 ± 13.5	0.575 ± 0.051	168 ± 25
	2	145 ± 3	0.452 ± 0.076	278 ± 59
Control (without enhancer)		0.781 ± 0.164	10.8 ± 0.1	0.679 ± 0.341

Each value represents the mean ± S.D. of three determinations.

1981). Results are given in Table 2. An impressive elevation in the D' value of ketoprofen was seen by increasing MET concentrations (0.5–1%) in the hydrogels. The K' value was remarkably enhanced when a small amount of MET (0.125%) was added to the hydrogels, while no further enhancement of the K' value was observed at higher concentrations of MET (0.25–2%). These results may suggest that the partitioning of ketoprofen from the hydrogel to the skin is improved by the addition of a small amount of MET, whereas the diffusivity of the drug is enhanced at higher concentrations of MET (0.5–1%). With lower concentrations of menthol (0.125–0.5%), the effect on permeation was negligible, compared with MET. More pronounced permeation, comparable with 0.25–0.5% MET, was only observed at higher concentrations of menthol (2%).

3.2. The effects of MET on the percutaneous absorption of ketoprofen

In order to evaluate the effects of MET in vivo, the apparent penetration rate (R_p) of ketoprofen was estimated from a simple pharmacokinetic model (Eq. (4)), based on the assumption that the penetration rate of ketoprofen absorbed from the hydrogel is constant after a lag time (Takayama and Nagai, 1991).

$$C = \frac{R_p}{V_d k_{10}}\left[1 + \frac{\beta - k_{10}}{\alpha - \beta}\exp(-\alpha(t - t_L)) + \frac{k_{10} - \alpha}{\alpha - \beta}\exp(-\beta(t - t_L))\right] \quad (4)$$

C is the plasma concentration, R_p is the apparent penetration rate, t is time, and t_L is the lag time, V_d is the distribution volume of the central compartment, k_{10} is the elimination rate constant from the central compartment, and α and β are the hybrid first-order rate constants. In a previous study, the plasma concentrations of ketoprofen after i.v. administration declined in a biexponential manner in rats, and pharmacokinetic parameters such as V_d, k_{10}, α and β were therefore calculated according to a two-compartment open model. The mean values of these parameters, estimated previously (Takayama and Nagai, 1991), were used in this study to determine R_p and t_L values. Furthermore, the area under the plasma concentration–time curve up to 8 h (AUC_{0-8h}) was estimated by the trapezoidal rule. Results are summarized in Table 3. When the hydrogel containing menthol was applied, R_p and AUC_{0-8h} values gradually increased as a function of the amount of menthol. However, prominent increases in both the R_p and AUC_{0-8h} values were observed in hydrogels containing a small amount of MET (0.25–0.5%). At least 2% menthol was required to obtain the same results. Furthermore, t_L values were greatly reduced as the R_p and

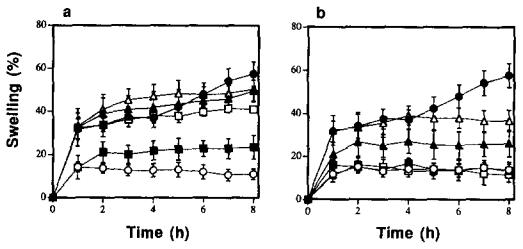

Fig. 3. Inhibitory effect of ketoprofen hydrogels containing menthol (a) or MET (b) on carrageenan-induced rat paw inflammation. Each point is the mean ± S.D. of six determinations. Concentrations of enhancer: (●) 0%; (△) 0.125%; (▲) 0.25%; (□) 0.5%; (■) 1%; (○) 2%.

$AUC_{0-8\,h}$ values increased. Results observed in the in vivo experiments broadly correlated with the results from the in vitro study, although a quantitative relationship between the in vivo parameters and the in vitro ones was not clear.

3.3. The effects of MET on the anti-inflammatory activity of ketoprofen

Fig. 3 shows the inhibitory effect of ketoprofen hydrogels containing menthol or MET on carrageenan-induced rat paw inflammation. The degree of swelling in the rat paw was defined as in the following equation.

$$\text{Swelling (\%)} = \frac{S_t - S_0}{S_0} \times 100 \quad (5)$$

S_t is the thickness of rat paw at time t after the irritant treatment and S_0 is the thickness before challenge of the irritant. When the hydrogel containing a small amount of menthol (0.125–0.5%) was applied, almost no inhibition on the swelling was observed. A significant inhibition, compared with control (without an enhancer), was seen in hydrogels containing 1–2% menthol. On the other hand, pronounced inhibitory action on the swelling came out in hydrogels containing a small amount of MET (0.25–0.5%). No further improvement was, however, seen at higher concentrations of MET (1–2%). Employing a Dunnet's multiple comparison test, we statistically analyzed the anti-inflammatory action of ketoprofen hydrogels containing menthol or MET. Results are given in Table 4. At least 1% menthol was required to exhibit significant inhibitory action of ketoprofen on the paw swelling. On the other hand, a small amount of MET (0.25–0.5%) was enough to bring about significant inhibitory action. The rat paw edema test revealed that the extent of anti-inflammatory action of ketoprofen percutaneously absorbed from hydrogels containing menthol or MET correlated well with the results from the in vitro permeation and the in vivo absorption studies.

Table 4
Dunnet's multiple comparison test for the inhibitory effect of ketoprofen hydrogels containing menthol or MET on carrageenan-induced rat paw inflammation

Enhancer	Concentration (%)	Time after administration (h)							
		1	2	3	4	5	6	7	8
Menthol	0.125	1.6	7.0	8.0	10.0	6.3	0.2	5.6	7.1
	0.25	0.9	5.0	3.7	4.7	1.4	3.0	8.2	7.9
	0.5	15.4	0.2	1.1	0.9	4.2	8.1	12.7	16.4*
	1	17.6*	12.7	17.3**	15.2*	19.6**	25.1**	31.2**	34.0**
	2	17.4*	20.2**	24.7**	24.3**	29.0**	35.9**	43.4**	46.5**
MET	0.125	0.5	0.5	2.3	1.9	4.5	10.1	17.6*	21.0*
	0.25	10.7	7.3	12.4	10.0	16.9	22.6*	28.4**	31.5**
	0.5	20.3*	17.9*	22.2**	23.8**	28.9**	34.3**	42.1**	45.9**
	1	15.5*	18.9**	23.7**	20.3**	28.1**	34.6**	39.4**	44.2**
	2	19.8**	18.8**	24.3**	22.9**	28.0**	34.2**	39.3**	43.8**

Each value represents the mean of % inhibition of swelling thickness from the control hydrogel without enhancer.
*$p<0.05$, **$p<0.01$.

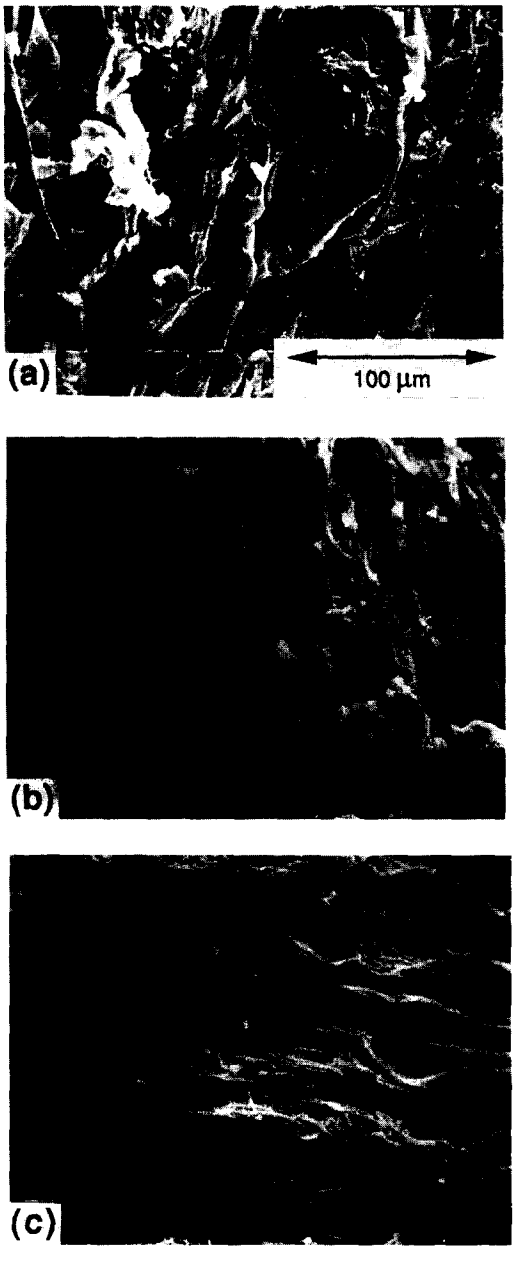

Fig. 4. Scanning electron micrographs of hairless rat skin treated with hydrogels containing menthol or MET for 8 h. Magnification: × 500. (a) Untreated; (b) treated with 0.5% MET; (c) treated with 2% menthol.

3.4. The effects of MET on the morphology of the skin surface

Morphological changes in the skin surface treated with hydrogels containing MET or menthol were examined by a scanning electron microscope. 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 in thick hair. Fig. 4 shows typical examples of microscopic photographs of the skin surface treated with hydrogels containing menthol or MET for 8 h. The intact skin surface (untreated with enhancers) was shown to have rough and irregular morphology (Fig. 4a). However, the roughness of the skin surface gradually decreased with an increase in the MET concentration. In particular, remarkable changes in the skin surface were observed with 0.5–2% MET, i.e. the spaces between the cells became extended and the shape of each cell became clear (Fig. 4b), whereas the morphological changes brought about by the treatment with menthol were relatively weak. Distinguishable changes similar to those observed with 0.5% MET were only seen after treatment with 2% menthol (Fig. 4c). 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). Therefore, MET and menthol would be distributed throughout the intercellular lipid region and may change the dense barrier structure of the stratum corneum. The effects of MET were significantly greater than those of menthol. The results of this study suggest that MET can be used to promote the percutaneous absorption of drugs.

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