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The objectives of our study were to evaluate the effect of four terpene enhancers, enhancer lipophilicity, and ethanol concentration using hydroxypropyl cellulose (HPC) and two Pluronic F-127 (PF-127) gel formulations on the percutaneous permeation of ketoprofen. All experiments were conducted using hairless mouse skin in vitro. Data recorded over 24 hr was compared with that for control gels (containing no terpene) using Franz diffusion cells. In the three gel formulations, the highest increase in the ketoprofen permeation was observed using limonene followed by nerolidol, fenchone, and thymol. Relationships were established between terpene lipophilicity, enhancement ratios for ketoprofen flux (ER_{flux}), and the cumulative amount of ketoprofen after 24 hr (Q_{24}) from the three gel formulations. However, no correlation was established between terpene lipophilicity and ketoprofen skin content values at 24 hr. Ethanol had a synergistic effect on the enhancing activity of the terpenes. Increasing the concentration of ethanol from 10% to 50% was associated with an increase in the permeation of ketoprofen. For example, use of PF-127 gel control (no terpene was included) containing 10% ethanol resulted in a ketoprofen flux of $19 \pm 2\ \mu g/cm^2\ h$ and $481 \pm 131\ \mu g/cm^2$ for Q_{24}. Furthermore, for PF-127 containing 33% ethanol the flux was $34 \pm 3\ \mu g/cm^2\ h$ and Q_{24} was $1420 \pm 111\ \mu g/cm^2$. However, HPC gel control that contained 50% ethanol resulted in a ketoprofen flux of $67 \pm 6\ \mu g/cm^2\ h$ and $2839 \pm 222\ \mu g/cm^2$ for Q_{24}.

Ketoprofen is a potent nonsteroidal anti-inflammatory drug (NSAID) with antipyretic properties that is widely used for the acute and long-term management of rheumatoid arthritis. However, the oral administration of this drug is usually accompanied by severe gastric and duodenal irritation (Lanza et al. 1998) and

because of its short half-life of 1.1–4 hr, it requires frequent oral dosage. Transdermal administration of ketoprofen would be a possible alternative offering distinct advantages, such as elimination of the variable rate of absorption and first-pass intestinal and hepatic metabolism inherent with oral dosing. Furthermore, this route provides sustained controlled drug administration at a zero order rate similar to that provided by intravenous (IV) infusion that is crucial for drugs with short half-lives. The transdermal route overcomes the risks and inconvenience associated with parenteral route use and eliminates many side effects from reduction of the peaks in drug plasma levels. As a result, patient compliance is improved (Southwell and Barry 1983; Barry 1987; Goodman and Barry 1988; Williams and Barry 1992).

Ultimately, the success of all transdermal systems depends on the ability of the drug to permeate the skin, in particular the stratum corneum which is recognized as the predominant diffusional barrier for drug permeation across the skin. To promote drug permeation across the skin, chemical penetration enhancers may be included in transdermal formulations. Many studies have evaluated such enhancers and their mechanisms of activity (Barry and Bennett 1987; Williams and Barry 1992; Aoyagi and Nagase 1995; Ogiso et al. 1995; Godwin and Michniak 1999; Kim et al. 1999).

Terpenes are natural volatile oils with low cutaneous irritancy and are therefore good candidates as skin penetration enhancers (Opdyke 1979). Monoterpenes, in particular, are effective penetration enhancers for both hydrophilic and lipophilic drugs (Okamoto et al. 1988; Godwin and Michniak 1999).

Enhancers and actives need to be placed in effective formulation bases. Hydrogels are such vehicles for the transdermal delivery of drugs and include hydroxypropyl cellulose (HPC) and Pluronics F-217 (PF-127). HPC is extensively used in oral and topical pharmaceutical formulations owing to its nontoxic and nonirritant properties (Wade and Weller 1995). In topical formulations, it is used as a thickening and viscosity-increasing agent, stabilizer, and emulsifying agent (Machida and Nagai 1974; Johnson et al. 1993). PF-127 is a nonionic surface active polyoxyethylene-polyoxypropylene copolymer that is used

primarily in pharmaceutical formulations as an emulsifying and solubilizing agent (Collett et al. 1979; Collett and Tobin 1979; El Shaboury 1989). It is reported to be nonirritant and nontoxic (Miyazakin et al. 1986; Gill et al. 1994). Unlike HPC, PF-127 has a sol-gel transition in the 25–35°C temperature range, resulting in thermogels (Saettone et al. 1988).

The objectives of our study were threefold: to evaluate the effect of terpene enhancers on the permeation of ketoprofen from gel formulations; to establish relationships between the terpene lipophilicity (fenchone log P 2.1 ± 0.3, thymol log P 3.28 ± 0.3, limonene log P 4.6 ± 0.2, and nerolidol log P 5.4 ± 0.4) and the various ketoprofen percutaneous parameters from the three gel formulations; and to investigate the effect of ethanol concentrations in the gel formulations on the permeation of ketoprofen across hairless mouse skin in vitro.

METHODS

Materials

Ketoprofen was provided by Sigma Chemical Co. (St. Louis, MO). Acetonitrile, methanol, and water used in the HPLC procedures were of analytical grade and purchased from EM Science (New Briggs, NJ). Aaper Alcohol and Chemical Co. (Shelbyville, KY) supplied the ethanol USP. Fenchone, thymol, d-limonene, and nerolidol were obtained from Aldrich Chemical Company (Milwaukee, WI). Glycerol, PF-127, and HPC were purchased from Spectrum Chemical Mfg. Corp. (Gardena, CA). Female hairless mice strain SKH1 (hr/hr), 6–8 weeks old were obtained from Charles River Lab. (Wilmington, MA). All used chemicals were without further purification.

Preparation of Gel Formulations

HPC gels were prepared using the recently reported method described by El-Kattan et al. (2000). The HPC gel composition is shown in Table 1. Briefly, ethanol was mixed with glycerol and distilled water. Then HPC was added slowly with continuous mixing until gel was formed. Then ketoprofen followed by terpene were added to the gel and mixed. The gels were left overnight at ambient temperature in a tightly sealed container.

TABLE 2
Composition (% w/w) of PF-127 gels

Component	Gel A % w/w	Gel B % w/w
PF-127	20	20
Purified water	80	50
Ethanol	—	30

Two PF-127 gels were prepared using the method described by Schmolka (1972). Gel A and Gel B compositions are shown in Table 2. For the preparation of Gel A, a weighed amount of PF-127 powder was mixed into cold water with agitation. For the preparation of Gel B, 30% alcohol solution was used instead of purified water. The mixture, following storage for 24 hr at 4°C, became a transparent solution. For the preparation of Gels C and D containing 10% ketoprofen, a mixture of 10% ketoprofen, 10% alcohol, and 2% terpene was prepared and thoroughly mixed in 78% PF-127 (using Gels A and B) in an ice-water bath and the solutions formed Gel C and D, respectively, at room temperature (Table 3).

In Vitro Skin Permeability Studies

Skin permeation of ketoprofen from gel formulations was measured using Franz diffusion cells (Permegear Inc., Riegelsville, PA). The effective permeation area of the diffusion cell and receptor cell volume were 0.64 cm^2 and 5.1 ml, respectively. The temperature was maintained at 37 ± 0.5°C. The receptor compartment contained isotonic phosphate buffer (pH 7.2) and 0.1% formaldehyde (37%) as a preservative and was constantly stirred at 600 rpm. Female hairless mouse skin (SKH1, Charles River Lab.) was mounted between the donor and receptor compartments. The 10% ketoprofen gel formulation (300 mg) was applied under occlusion to the epidermal surface of the mouse skin. A portion of the receptor solution (300 μl) was collected through the sampling port of the diffusion cell at predetermined time intervals over 24 hr. The receptor phase was immediately replenished with equal volume of fresh diffusion buffer. Samples were frozen at −70°C before HPLC

TABLE 1
Composition (%w/w) of ketoprofen HPC gel

Component	% w/w
Ketoprofen	10
Ethanol	50
Water	26
Glycerol	10
Terpene	2
HPC	2

TABLE 3
Composition (% w/w) of ketoprofen PF-127 gels

Component	Gel C % w/w	Gel D % w/w
Ketoprofen	10	10
Ethanol	10	10
Terpene	2	2
Gel A	78	—
Gel B	—	78

analysis. After 24 hr, the skins were removed from the cells and washed briefly in methanol using previously reported procedures (Michniak et al. 1994). The skins were then homogenized in 4 ml methanol using a Kinematica GmbH tissue homogenizer. The homogenates were filtered and stored at −70°C until analyzed using HPLC (Michniak et al. 1993).

HPLC Analysis of Ketoprofen

The liquid chromatograph used (Hewlett Packard 1100 HPLC) was equipped with a quaternary pump with a variable–wavelength detector operating at 242 nm and an autosampler with an injection volume of 20 μl. Analysis was performed at ambient temperature on a C_{18}-Microsorb column (15 cm × 4.6 mm and 5 μm diameter). The mobile phase consisting of ace-tonitrile: 60 mM KH_2PO_4: triethanolamine (49:51:0.05), was pumped at a flow rate of 1 m/1 min. Calibrations were carried out by the external standard methods.

Data Analysis

In vitro percutaneous permeation parameters were calculated using equation 1.

$$J_t = (1/A)(dM/dt) \qquad [1]$$

where J_t is the flux (μg/cm^2 hr), dM/dt is the slope of the cumulative corrected drug amount in the receptor compartment versus time, and A is the diffusional area (cm^2).

The steady-state flux, J_t (μg/cm^2 hr) was determined from the slope of the linear portion of the cumulative amount permeated per unit area versus time plot. The lag time was determined by extrapolating the linear portion of the curve to the abscissa. *Log* P values of terpene enhancers were determined using ACD software program (Advanced Chemistry Incorporated, Ontario, Canada). Enhancement ratios for flux (ER$_{flux}$) were calculated using the following equation:

$$ER_{flux} = \frac{\text{Ketoprofen flux with enhancer in gel}}{\text{Ketoprofen flux without enhancer in gel (control)}} \qquad [2]$$

The data are presented as means ± *SD* of five experiments. Statistical analyses were performed using one-way ANOVA. Correlation analyses were performed by the least squares linear regression method.

RESULTS

Effect of HPC Formulations on the Percutaneous Permeation of Ketoprofen

The effect of terpene enhancers on the percutaneous permeation profiles of ketoprofen is depicted in Figure 1. The flux, ER$_{flux}$, cumulative amount of ketoprofen after 24 hr (Q$_{24}$), and ketoprofen skin content (SC) are shown in Table 4. Controls consisted of gel formulations with no terpene incorporation and the
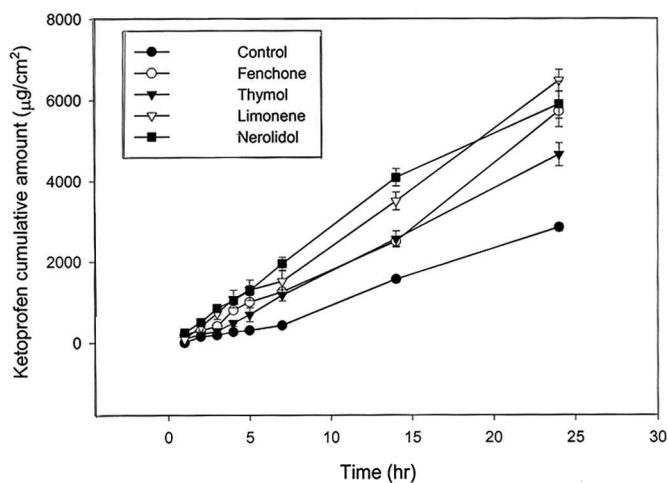

FIG. 1. The permeation profile for ketoprofen from the HPC gel system using terpene enhancers. Means ± SE, $n = 5$.

flux recorded was 67 ± 6 μg/cm^2 hr, Q$_{24}$ was 2839 ± 222 μg/cm^2, and 9384 ± 1789 μg/g for SC. Terpene enhancers had a significant effect on the percutaneous permeation parameters of ketoprofen across the female hairless mouse skin in relation to control ($p < .05$) (Table 4). ER$_{flux}$ using limonene (4.5) was not significantly different from that for nerolidol (4.4) and fenchone (3.7) ($p > .05$); however, it was significantly higher than that observed with thymol (3.1) ($p < .005$) and control ($p < .001$). The use of limonene resulted in the highest increase in Q$_{24}$ (6473 ± 539 μg/cm^2) that was significantly higher than control ($p < .0005$). However, it was not significantly different from nerolidol (5887 ± 1120 μg/cm^2) and fenchone (5416 ± 1042 μg/cm^2) ($p > .05$). Thymol provided the lowest increase in Q$_{24}$ (4649 ± 574 μg/cm^2), yet it was significantly higher than that provided by control gel ($p < .01$).

The highest ketoprofen skin content was observed with fenchone (16926 ± 578 μg/g) followed by limonene (14086 ± 934 μg/g), thymol (13539 ± 5443 μg/g), and nerolidol (11184 ± 2557 μg/g).

TABLE 4
Effect of terpenes on ketoprofen percutaneous permeation from HPC ketoprofen gel ($n = 5$) (means ± *SD*)

Enhancer	Flux (μg/cm^2 hr)	ER$_{flux}$	Q$_{24}$ (μg/cm^2)	SC (μg/g)
Control	67 ± 6	1.0	2839 ± 222	9384 ± 1789
Fenchone	248 ± 39	3.7	5416 ± 1042	16926 ± 578
Thymol	207 ± 23	3.1	4649 ± 574	13539 ± 5443
Limonene	304 ± 14	4.5	6473 ± 539	14086 ± 934
Nerolidol	297 ± 23	4.4	5887 ± 1120	11184 ± 2557

SC: skin content of ketoprofen after 24 hr; Q$_{24}$: cumulative amount of ketoprofen in the receptor after 24 hr; ER$_{flux}$: enhancement ratio of ketoprofen flux.

TABLE 5
Effect of terpenes on ketoprofen percutaneous permeation
from ketoprofen PF-127 Gel *C* (*n* = 5) (means ± *SD*)

Enhancer	Flux (μg/cm^2 hr)	ER$_{flux}$	Q$_{24}$ (μg/cm^2)	SC (μg/g)
Control	19 ± 2	1.0	481 ± 131	11424 ± 3015
Fenchone	84 ± 11	4.4	1770 ± 243	6512 ± 1551
Thymol	66 ± 17	2.9	1441 ± 189	7158 ± 727
Limonene	121 ± 16	6.2	2592 ± 508	5670 ± 2534
Nerolidol	104 ± 22	5.5	2289 ± 622	12804 ± 2692

SC: skin content of ketoprofen after 24 hr; Q$_{24}$: cumulative amount of ketoprofen in the receptor after 24 hr; ER$_{flux}$: enhancement ratio of ketoprofen flux.

Effect of Pluronic Acid Gel Formulations on the Percutaneous Permeation of Ketoprofen

Two PF-127 gel formulations were prepared with different ethanol contents (Gels *C* and *D*, Table 3). The effect of terpene enhancers on the percutaneous permeation parameters of ketoprofen using Gels *C* and *D* is shown in Tables 5 and 6, respectively. Similar to the data obtained with HPC gel formulations, the terpenes had a pronounced effect on the percutaneous permeation parameters of ketoprofen (Figures 2 and 3).

In Gel *C*, limonene showed the best enhancing effect in terms of ketoprofen ER$_{flux}$ (6.2), which was significantly higher than control ($p < .001$). However, it was not significantly different from the ER$_{flux}$ provided by nerolidol (5.5) and fenchone (4.4) ($p > .05$). Thymol provided the lowest increase in the ER$_{flux}$ (2.9), which was significantly lower than limonene ($p < .05$) yet higher than control ($p < .05$). In addition to providing the highest ER$_{flux}$, limonene provided the highest increase in Q$_{24}$ (2592 ± 508 μg/cm^2) in relation to control (481 ± 131 μg/cm^2) ($p < .01$). Nerolidol and fenchone increased Q$_{24}$ to 2289 ± 622 μg/cm^2 and 1770 ± 243 μg/cm^2, respectively. However, they were not significantly different from limonene ($p > .05$). The lowest Q$_{24}$ was provided by thymol (1441 ± 189 μg/cm^2). The highest ke-

TABLE 6
Effect of terpenes on ketoprofen percutaneous permeation
from ketoprofen PF-127 Gel *D* (*n* = 5) (means ± *SD*)

Enhancer	Flux (μg/cm^2 hr)	ER$_{flux}$	Q$_{24}$ (μg/cm^2)	SC (μg/g)
Control	34 ± 3	1.0	1420 ± 111	9850 ± 3300
Fenchone	141 ± 53	4.1	3228 ± 1191	13812 ± 3147
Thymol	110 ± 19	3.2	2717 ± 215	13586 ± 2531
Limonene	256 ± 23	7.5	5932 ± 434	5932 ± 434
Nerolidol	242 ± 28	7.1	4956 ± 373	13875 ± 4632

SC: skin content of ketoprofen after 24 hr; Q$_{24}$: cumulative amount of ketoprofen in the receptor after 24 hr; ER$_{flux}$: enhancement ratio of ketoprofen flux.
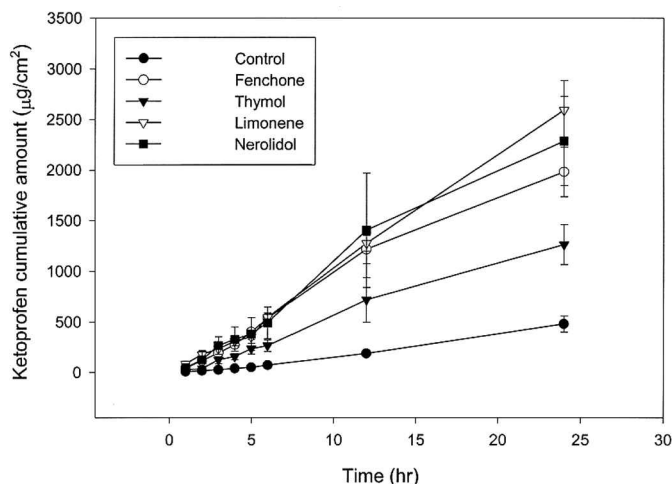

FIG. 2. The permeation profile for ketoprofen from the PF-127 Gel *C* system using terpene enhancers. Means ± SE, *n* = 5.

toprofen skin content was observed with nerolidol (12804 ± 2692 μg/g) which was not significantly higher than the control gel (11424 ± 3015 μg/g) ($p > .05$). Thymol, fenchone, and limonene provided ketoprofen skin contents (7158 ± 727 μg/g, 6512 ± 1551 μg/g, and 5670 ± 2534 μg/g, respectively) that were not significantly lower than control ($p < .05$).

In Gel *D*, limonene also provided the best enhancing activity in terms of ketoprofen ER$_{flux}$ (7.5), which was significantly higher than control ($p < .0001$). However, it was not significantly different than the ER$_{flux}$ provided by nerolidol (7.1) ($p > .05$). Fenchone and thymol provided 4.1 and 3.2 increase in ER$_{flux}$ which were significantly lower than limonene ($p < .025$) and ($p < .001$), respectively, yet higher than control ($p < .002$) and ($p < .0001$), respectively. Similar to the results obtained with HPC gel and Gel *D*, limonene provided the highest increase in Q$_{24}$ (5932 ± 434 μg/cm^2) compared with control
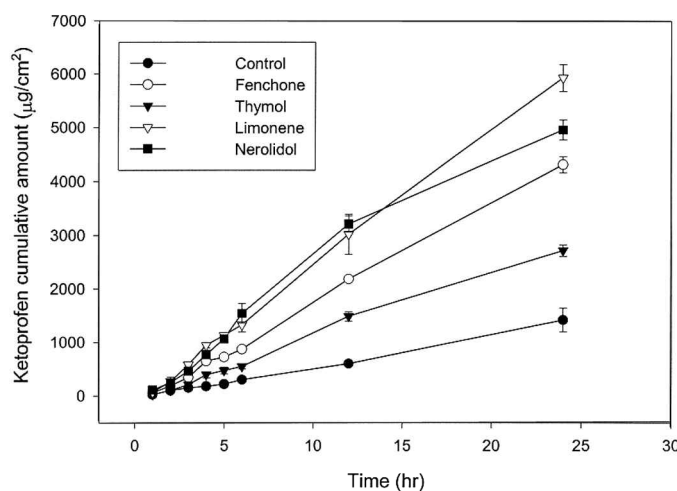

FIG. 3. The permeation profile for ketoprofen from the PF-127 Gel *D* system using terpene enhancers. Means ± SE, *n* = 5.

$(1420 \pm 111 \ \mu g/cm^2)$ $(p < .00025)$. Nerolidol increased Q_{24} to $4956 \pm 373 \ \mu g/cm^2$; however, it was not significantly different from limonene $(p > .05)$. Fenchone increased Q_{24} to $3228 \pm 1191 \ \mu g/cm^2$ that was significantly lower than limonene $(p > .01)$. Thymol provided the lowest increase in Q_{24} $(2717 \pm 215 \ \mu g/cm^2)$ relative to control gel. The highest ketoprofen skin content was observed with nerolidol $(13875 \pm 4632 \ \mu g/g)$, followed by thymol $(13586 \pm 2531 \ \mu g/g)$, and fenchone $(13812 \pm 3147 \ \mu g/g)$. They were not significantly higher than control $(9850 \pm 3300 \ \mu g/g)$ $(p > .05)$. The lowest ketoprofen skin content was provided by limonene $(5932 \pm 434 \ \mu g/g)$, and it was not significantly lower than control $(p > .05)$.

DISCUSSION

In our study, the effect of four terpene enhancers (fenchone, thymol, limonene, and nerolidol) on the percutaneous permeation of ketoprofen was evaluated from three gel formulations using hairless mouse skin in vitro. Hairless mouse skin was used as a model skin although it does provide higher permeability than human skin. The basic mechanism of permeation, however, is thought to be similar between the two species (Ghosh and Bagherian 1996; Gorukanti et al. 1999). A number of research groups independently investigated the mechanism of terpene permeation enhancement activity using differential scanning calorimetry and Fourier transform infrared (Cornwell and Barry 1991, 1994; Zhao and Singh 1999). They found that terpenes increased the drug percutaneous permeation mainly by disrupting the highly ordered intercellular packing of the stratum corneum lipids.

Terpene enhancers had a significant effect on the percutaneous permeation of ketoprofen from the evaluated gel formulations. Furthermore, the results suggested that limonene, a hydrocarbon terpene, was more effective in promoting the percutaneous permeation of ketoprofen (lipophilic drug with a log P of 2.81 ± 0.33) from the gels, when compared with that provided by polar terpenes such as thymol, fenchone, and nerolidol. The data obtained were similar to those reported by other research groups (Koyama et al. 1994; Ogiso et al. 1995; Moghimi et al. 1998; Godwin and Michniak 1999; Kitagawa and Li 1999). Koyama and coworkers (1994) investigated the enhancing effect of limonene on the percutaneous permeation of mannitol (hydrophilic drug), 6-mercaptopurine (amphiphilic drug), and butylparaben (lipophilic drug) using guinea pig skin in vitro. They reported that limonene had a significant effect on the percutaneous permeation of butylparaben and 6-mercaptopurine but had a little effect on the mannitol percutaneous permeation (Koyama et al. 1994). They attributed the enhancing activity of limonene to its ability to increase drug diffusivity by disrupting the normal packing of the skin. Furthermore, limonene decreased lipophilic drug partitioning in the nonpolar route leading to an acceleration of its penetration. Yamane et al. (1995) evaluated the effect of 1,8 cineole, menthone, nerolidol, and limonene in propylene glycol/water on the permeation of a model

hydrophilic permeant, 5-fluorouracil, using excised human skin in vitro. 1,8 cineole, menthone, and nerolidol increased the permeation of 5-fluorouracil significantly through disrupting the lipid packing of the stratum corneum or by increasing the drug partitioning. However, limonene did not significantly increase 5-fluorouracil permeation.

In our study, nerolidol was the most effective penetration enhancer compared with fenchone and thymol. Its high enhancing activity has been previously reported and is attributed to its amphiphile-like structure that may lead to the disruption of the lipid packing of the stratum corneum (Cornwell and Barry 1994a, 1994b; Yamane et al. 1995).

It is interesting to note that the increase in the lipophilicity of the terpene was associated with a proportional increase in the percutaneous permeation of ketoprofen. The correlation coefficient between the lipophilicity of terpene and ER_{flux} was 0.736 in HPC gel, 0.652 in PF-127 Gel C, and 0.82 in PF-127 Gel D. Furthermore, the correlation coefficient between the log P of terpene enhancers and Q_{24} was 0.60 in HPC gel, 0.706 in PF-127 Gel C, and 0.76 in PF Gel D. No correlation was established between the log P of the terpene enhancers and the ketoprofen skin contents using the three gel formulations. Similar results were reported by El-Kattan et al. (2000). A correlation coefficient was established between the terpene log P and the flux of hydrocortisone, and log P and cumulative amount of hydrocortisone after 24 hr (0.891 and 0.772, respectively). No correlation was found between the log P of terpene and hydrocortisone skin content.

In our present study, the formulations containing HPC provided the highest increase in the percutaneous permeation of ketoprofen relative to the PF-127 formulations. This effect may be attributed to the higher ethanol concentration found in the HPC formulations (Table 1). Decreasing the concentration of ethanol from 50% to 33% and to 10% when using HPC gel, PF-127 Gel D and C, respectively, was associated with a decrease in the ketoprofen percutaneous permeation. The synergistic effect of ethanol on the enhancing activity of terpene enhancers was evaluated previously (Kikuchi et al. 1992; Priborsky et al. 1992; Maitani et al. 1996; Zhao and Singh 1999). Okabe et al. evaluated the effect of limonene and ethanol on the percutaneous permeation of ketoprofen from acrylic gel patches using rat skin in vivo. They demonstrated that limonene did not provide any enhancing activity for ketoprofen permeation when ethanol was not used. Furthermore, when ethanol was combined with limonene and a hydrophilic acrylic polymer, the permeability of ketoprofen was increased significantly suggesting that ethanol is an essential adjuvant to obtain the activity of d-limonene. We reported earlier the effect of ethanol and glycerol concentrations on the percutaneous permeation of hydrocortisone from HPC gel formulations (El-Kattan et al. 1999). We showed that increasing ethanol concentration from 0% to 56% to 85% was associated with a significant increase in the permeation of hydrocortisone. On the other hand, glycerol concentrations did not have a significant effect on the percutaneous permeation of

hydrocortisone at a concentration range of 0–10%. At 35% glycerol, the permeation of hydrocortisone decreased significantly and was attributed to the hygroscopic properties of glycerol at high concentrations. Kobayashi et al. (1994) reported that the simultaneous use of menthol and ethanol affected the inherent enhancing activity of the two enhancers. In addition, they suggested that the enhancing effect was synergistic. However, the mechanisms of enhancement of both enhancers were different, with ethanol providing enhancing activity by increasing porosity of the stratum corneum and menthol by promoting drug diffusion through disrupting the lipid backing of the skin.

CONCLUSION

Terpene enhancers had a significant effect on the percutaneous permeation of ketoprofen from gel formulations. Limonene showed the highest enhancement of ketoprofen permeation compared with other terpene enhancers and control followed by nerolidol, fenchone, and thymol. The increase in terpene enhancer lipophilicity was associated with a proportional increase in the ER_{flux} and Q_{24}. No correlation was established between terpene lipophilicity and ketoprofen skin contents provided by the three formulations. Ethanol is an important adjuvant for the proper enhancing activity of the terpene enhancers and increasing its concentration was associated with an increase in permeation of ketoprofen across the skin.

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