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Mechanism of transdermal transport of 5-fluorouracil by terpenes: carvone, 1,8-cineole and thymol

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Abstract

The effect of terpenes as penetration enhancers (e.g. carvone, 1,8-cineole and thymol) was studied on the in vitro percutaneous absorption of the model hydrophilic compound 5-fluorouracil through porcine epidermis. The above terpenes (5% w/v) significantly ($P < 0.01$) increased the permeability coefficient of 5-fluorouracil in comparison to the control. Enhancement in the permeability of 5-fluorouracil by carvone, 1,8-cineole and thymol in comparison to the control was 91.62, 153.75 and 273.75, respectively. Fourier transform infrared (FT-IR) spectroscopy, and in vitro transepidermal water loss (TEWL) studies were undertaken to investigate the effect of enhancers on the biophysical properties of the stratum corneum and macroscopic barrier integrity of the epidermis, respectively, in order to understand the mechanism of percutaneous absorption enhancement of 5-fluorouracil by terpenes. The FT-IR spectrum of the stratum corneum treated with thymol produced a blue shift in the antisymmetric C–H stretching peak to higher wavenumbers, suggesting an increase in the disorder of the acyl chains of the stratum corneum lipids (i.e. increased lipid fluidity). Treatments of the epidermis with enhancers significantly ($P < 0.01$) enhanced the in vitro TEWL in comparison to the control. © 1997 Elsevier Science B.V.

Keywords: 5-Fluorouracil; Fourier transform infrared; Penetration enhancer; Percutaneous absorption; Terpenes; Transepidermal water loss

1. Introduction

Various studies have demonstrated that the transdermal pathway may be a suitable alternative to the oral route in the administration of drugs with systemic activity. The advantages provided by the dosing of drugs in transdermal delivery systems are well known (Wester and Maibach, 1992). Thus, drugs with a high intrinsic pharmacological activity that are used in pathologies that require relatively long-term therapy are potentially suitable for formulation in transdermal delivery systems.

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The basic data for in vivo human percutaneous absorption, with which animal models are compared, were obtained from Feldmann and Maibach (1969a,b, 1974). The ranking of skin permeability of different species in vitro have been determined by several investigators (Tregear, 1966; Marzulli et al., 1969; Wester and Maibach, 1989). Increasing evidence supports the contention that in vitro permeability studies can accurately predict in vivo absorption (Bronaugh, 1989). Skin from the pig generally approximates the permeability of human skin (Bhatia and Singh, 1996).

The primary barrier to transdermal diffusion is the stratum corneum, the thin outermost layer of the skin that is comprised of a regular array of protein-rich cells embedded in a multilamellar lipid domain. The lamellar packing of stratum corneum intercellular lipids is established and several experiments have directly implicated these lipoidal domains as the integral components of the transport barrier (Sweeny and Downing, 1970). Many strategies have been suggested to overcome skin impermeability, such as iontophoresis (Singh and Bhatia, 1996), or the application of supersaturated drug systems (Davis and Hadgraft, 1991). A popular technique is the use of penetration enhancers which reversibly reduce the permeability barrier of the stratum corneum (Barry, 1983). Over the last two decades, much research has focused on the enhancing ability of a wide range of substances and their mechanisms of action (as recently reviewed by Williams and Barry (1992)).

Generally, the modes of action of skin penetration enhancers involve increasing drug diffusivity through the skin by affecting the barrier properties of the stratum corneum (Goodman and Barry, 1988), and/or increasing the partitioning of the drug into the stratum corneum (Barry, 1988; Okamoto et al., 1988; Sasaki et al., 1991). A more recently suggested mode of action is one of phase separation, for molecules such as oleic acid within the stratum corneum lipid bilayers (Walker and Hadgraft, 1991).

Terpene is a constituent of an essential oil and consists of isoprene (C_5H_8) units. Despite the widespread medicinal usage of many terpenes, there are few reports of their penetration-enhancing properties. 1,8-Cineole has been used to promote the percutaneous absorption of several lipophilic drugs through hairless mouse skin (Zupan, 1982). Patents exist for the use of 1-carvone and eugenol as skin penetration enhancers (Leonard et al., 1989). Terpenes containing 50% ethanol increased the total flux of nicotine through the hairless-mouse skin (Nuwayser et al., 1988). Some monocyclic monoterpenes such as limonene and menthol enhanced the transport of indomethacin (Okabe et al., 1989) and diazepam (Hori et al., 1991), respectively, through the rat skin. Thymol is incorporated in some lotions and creams. It is also included in topical mixtures as a counterirritant and in mouth washes for its antiseptic action. Currently, natural products are receiving considerable interest in the pharmaceutical industry, and terpenes may provide a series of relatively safe, clinically acceptable accelerants for lipophilic and hydrophilic drugs.

Fourier transform infrared (FT-IR) spectroscopy provides information on the vibrational modes of its components and hence probes the structure on a molecular level. Transepidermal water loss (TEWL) provides a robust method for assessing macroscopic changes in the barrier properties of the skin (Abrams et al., 1993). The ability of different solvents to induce changes in

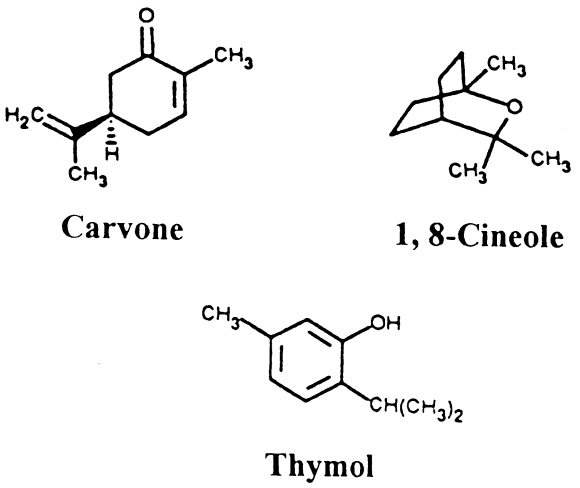

Fig. 1. Structural formula of terpenes used in the percutaneous absorption of 5-fluorouracil.

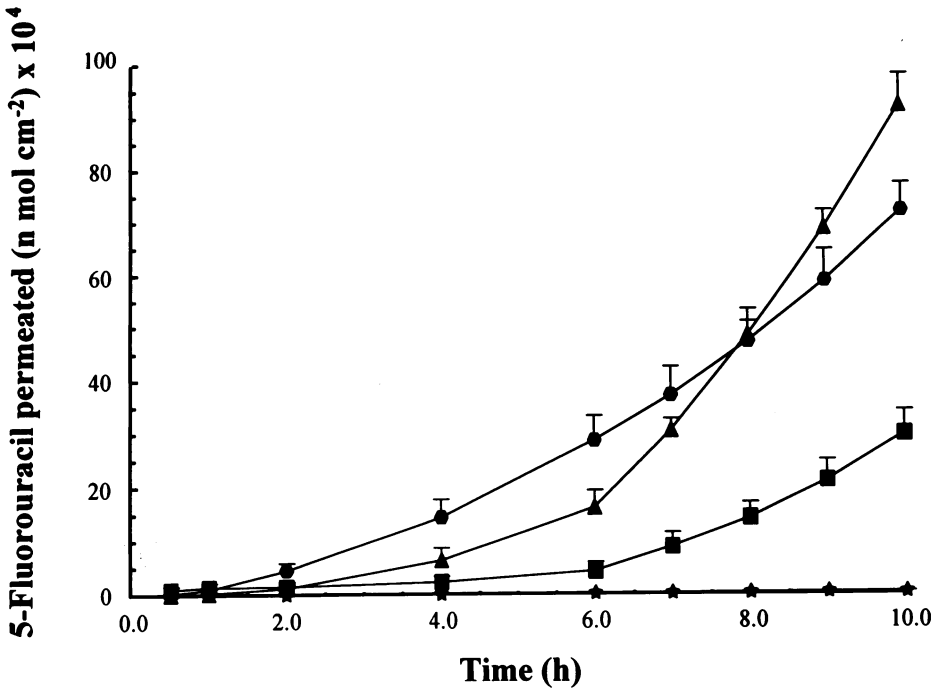

Fig. 2. The effect of terpenes on the in vitro transport of 5-fluorouracil through porcine epidermis. Each data point is the mean ± S.D. of three determinations. Key: (✱) control; (●) cineole; (■) carvone; (▲) thymol.

the skin's barrier function has been assessed in vitro by comparing pre- to post-solvent exposure TEWL (Abrams et al., 1993). Biophysical evidence suggests that stratum corneum lipid domains are the primary barrier to water loss and to penetration of compounds into the skin (Van Duzee, 1975). In addition, removal of lipids from the stratum corneum by solvent extraction leads to a 100-fold increase in water permeability (Scheuplein and Blank, 1971). Thus, the role of stratum corneum lipids in regulation water loss is well established (Knutson et al., 1985). The mechanism by which water passes through the stratum corneum, or any other lamellar lipid phase, is not well characterized. It has been suggested that water permeates through lipid lamellae via free-volume voids created due to random fluctuations in alkyl chain packing (Golden et al., 1987a). In this study, we selected three simple cyclic terpenes (e.g. carvone, 1,8-cineole and thymol) from the chemical classes of ketones, oxides and alcohols (Fig. 1) to investigate their effects on the biophysical changes in the stratum corneum lipids and on the macroscopic barrier integrity of the epidermis, and correlated these changes with the in vitro percutaneous absorption of 5-fluorouracil.

2. Materials and methods

2.1. Materials

[^{3}H]5-Fluorouracil (specific activity 23.1 Ci/mmol) was obtained from Moravek Biochemicals, CA. Carvone was purchased from Aldrich Chemical Company, Milwaukee, WI. 1,8-Cineole and thymol were purchased from Sigma Chemical, St. Louis, MO. Ethanol was purchased from CMS, TX. All other chemicals and reagents used were of analytical grade.

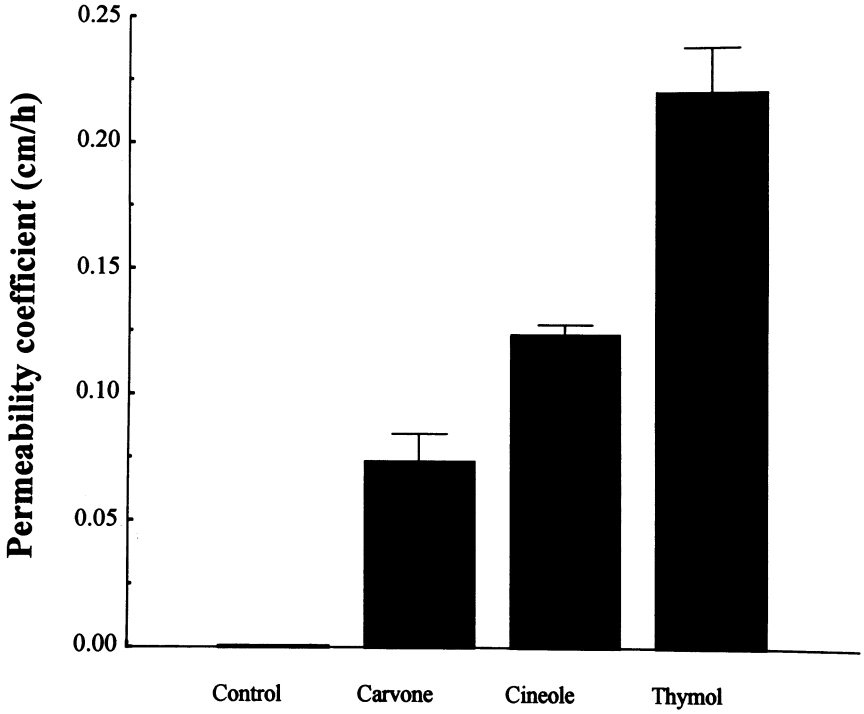

Fig. 3. The effect of terpenes on the permeability coefficient of 5-fluorouracil through porcine epidermis.

2.2. Preparation of epidermis

Porcine ears were obtained from a local slaughterhouse. Epidermal membranes were prepared by the heat-separation technique (Kligman and Christophers, 1963; Williams and Barry, 1991b). The whole skin was soaked in water at 60°C for 45 s, followed by careful removal of the epidermis. The epidermis was then washed with water and used in in vitro transport studies.

2.3. Preparation of stratum corneum

The epidermis was incubated for 4 h in a 0.5% trypsin solution in phosphate-buffered saline (pH 7.4) at 37°C. The tissue was then smoothed out on a flat surface and the mushy epidermis removed by rubbing with a moistened cotton tipped applicator. The transparent stratum corneum obtained was briefly floated on water and lifted out on aluminum foil, blotted dry, and used in FT-IR spectroscopic studies.

2.4. In vitro percutaneous absorption

Franz diffusion cells were used in all in vitro percutaneous absorption studies. The epidermis was sandwiched between the cells with the stratum corneum facing the donor compartment. The maximum capacity of each of the donor and receiver compartments was 2 and 5 ml, respectively. The surface of the epidermis exposed to the solution was 0.785 cm^2. The donor compartment contained 1 ml of 5-fluorouracil solution (0.2 μCi of 5-fluorouracil contained in a 1 ml mixture of 5% terpene in 50% ethanol) and the receiver compartment contained 5 ml of phosphate-buffered saline (pH 7.4). We used 50% ethanol in water in donor solution to solubilize the terpenes. The cells were maintained at 37 ± 0.5°C by PMC Dataplate® stirring digital dry block heater (Crown Bioscientific, NJ). The content of the receiver compartment was stirred with the help of a magnetic bar at 100 rpm. At specified intervals, 0.5 ml samples were withdrawn from the receiver com-

Table 1
Comparison of reported permeability coefficient and enhancement factor of 5-fluorouracil through the epidermis

Terpenes	Permeability coefficient (cm/h)		Enhancement factor	Skin type	Reference
	Control	Treated			
Carvone	1.29×10^{-5}	15.9×10^{-5}	12.3	Human	Williams and Barry (1991b)
Cineole	2.15×10^{-5}	204×10^{-5}	94.8	Human	Williams and Barry (1991b)
Carvone	0.8×10^{-3}	73.3×10^{-3}	91.62	Pig	This work
Cineole	0.8×10^{-3}	123.0×10^{-3}	153.75	Pig	This work

partment and an equivalent amount of phosphate-buffered saline (0.5 ml) was added to maintain the constant volume. Appropriate control experiments were also performed without terpenes, i.e. using 0.2 μCi/ml of 5-fluorouracil in 50% ethanol as donor solution.

The samples were assayed for 5-fluorouracil contents by liquid scintillation counting. Each sample was mixed with 10 ml of scintillation cocktail (Econosafe®, biodegradable counting cocktail, Research Products International, IL), and counted in a liquid scintillation counter (Packard, Tri Carb® 2100 TR, CT). The instrument was programmed to give counts for 10 min. The results were expressed as the mean $\pm$ S.D. of three experiments.

2.5. Fourier transform infrared (FT-IR) spectroscopy

The stratum corneum was soaked in enhancer solution for 2 h, washed with water and blotted dry. The stratum corneum was then subjected to FT-IR studies. The spectrum was obtained in the frequency range 4000–1000 cm^{-1}. Attention was focused on characterizing the occurrence of peaks near 2850 and 2920 cm^{-1} which were due to the symmetric and asymmetric C–H stretching, respectively. Of particular interest was a shift of the C–H stretching absorbances to higher wavenumbers. FT-IR (2020 Galaxy Series FT-IR, Mattson Instrument, Madison, WI) was used to accomplish this study. FT-IR experiments with each condition were performed in triplicate.

2.6. In vitro transepidermal water loss (TEWL)

Franz-type diffusion cells were used for in vitro TEWL studies. The epidermis was soaked in the enhancer solution for 2 h. The epidermis was then sandwiched between the diffusion cells with the stratum corneum side up and the dermal side exposed to the receiver compartment containing isotonic saline (0.9% sodium chloride solution). The surface area of the epidermis exposed for TEWL was 0.785 cm^2. The temperature of the diffusion cells was maintained at 37 ± 0.5°C. The epidermis was allowed to equilibrate in the in vitro system for 4 h before TEWL measurements with Tewameter™. TEWL measurements were performed by holding the Tewameter™ probe over the donor cell opening until a stable TEWL value was achieved. The experiments were performed in a room with an ambient temperature between 20 and 26°C and relative humidity between 46 and 58%. All experiments were performed in triplicate, and the results expressed as mean $\pm$ S.D. Experiments were performed in the same manner without enhancer treatment of the epidermis to serve as control.

2.7. Data analysis

The cumulative amount of solute permeated per unit skin surface area was plotted against time and the slope of the linear portion of the plot was estimated as steady-state flux (J_{ss}). The permeability coefficient (K_p) was calculated as (Scheuplein, 1978):

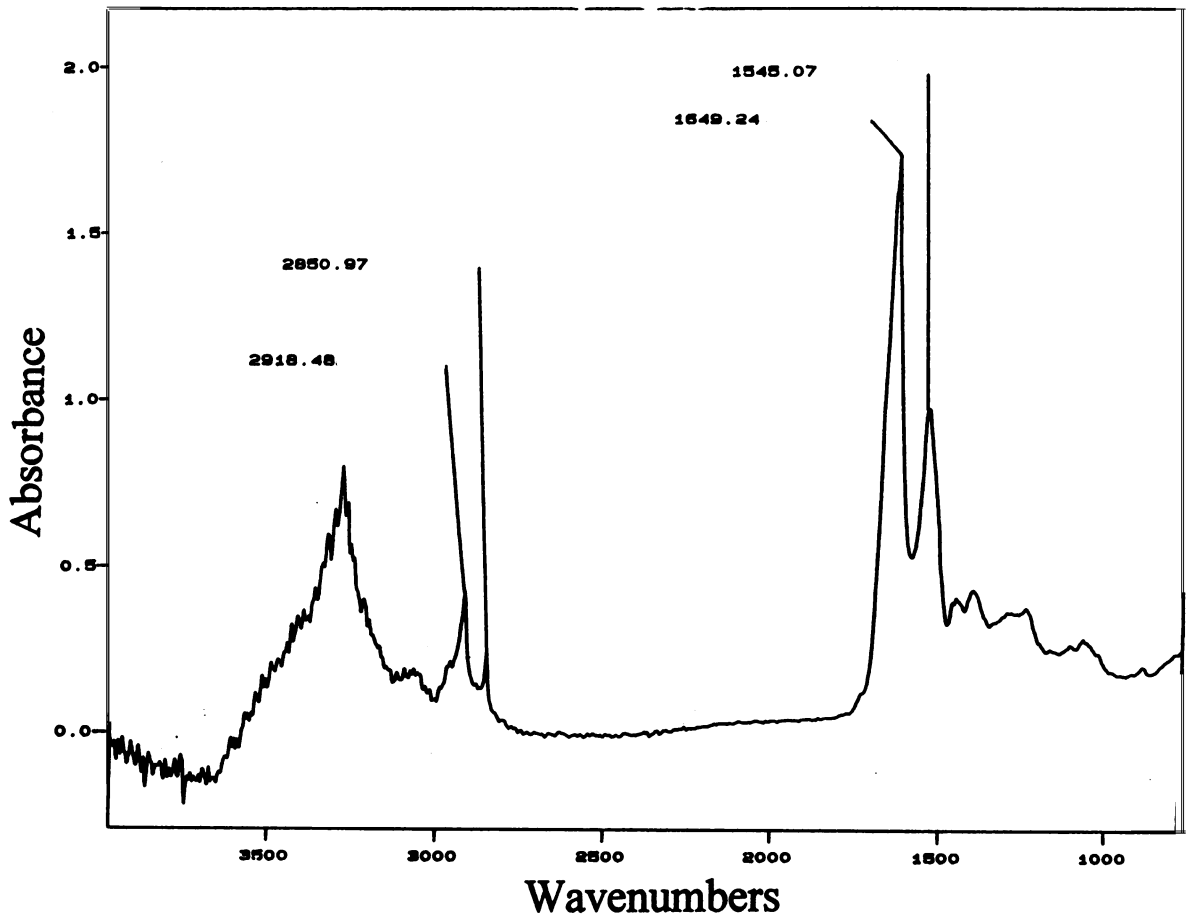

Fig. 4. FT-IR spectrum of porcine stratum corneum treated with 50% ethanol.

$$K_p = \frac{J_{ss}}{C_v}$$

where C_v is the total donor concentration of the solute. The C_v of 5-fluorouracil used was 8.65×10^{-3} nmol/ml. Statistical comparisons were made using Student's t-test. The level of significance was taken as $P < 0.05$.

3. Results and discussion

The effect of terpenes (carvone, 1,8-cineole and thymol) on the in vitro percutaneous absorption profiles of 5-fluorouracil through porcine epidermis is shown in Fig. 2. The above terpenes increased the in vitro transport of 5-fluorouracil as compared to the control. The permeability coefficients of 5-fluorouracil through the epidermis are shown in Fig. 3. The permeability coefficient of 5-fluorouracil in the presence of terpenes was significantly higher ($P < 0.01$) in comparison to the control. The enhancement factors (i.e. ratio of the permeability of 5-fluorouracil with terpene to the permeability without terpene) for carvone, 1,8-cineole and thymol were 91.62, 153.75 and 273.75, respectively. Thus, thymol produced greater enhancement in the permeability of 5-fluorouracil through the porcine epidermis followed by 1,8-cineole and carvone. Williams and Barry (1991a) found that hydrocarbon terpenes show minimal activity as compared to the oxygen-

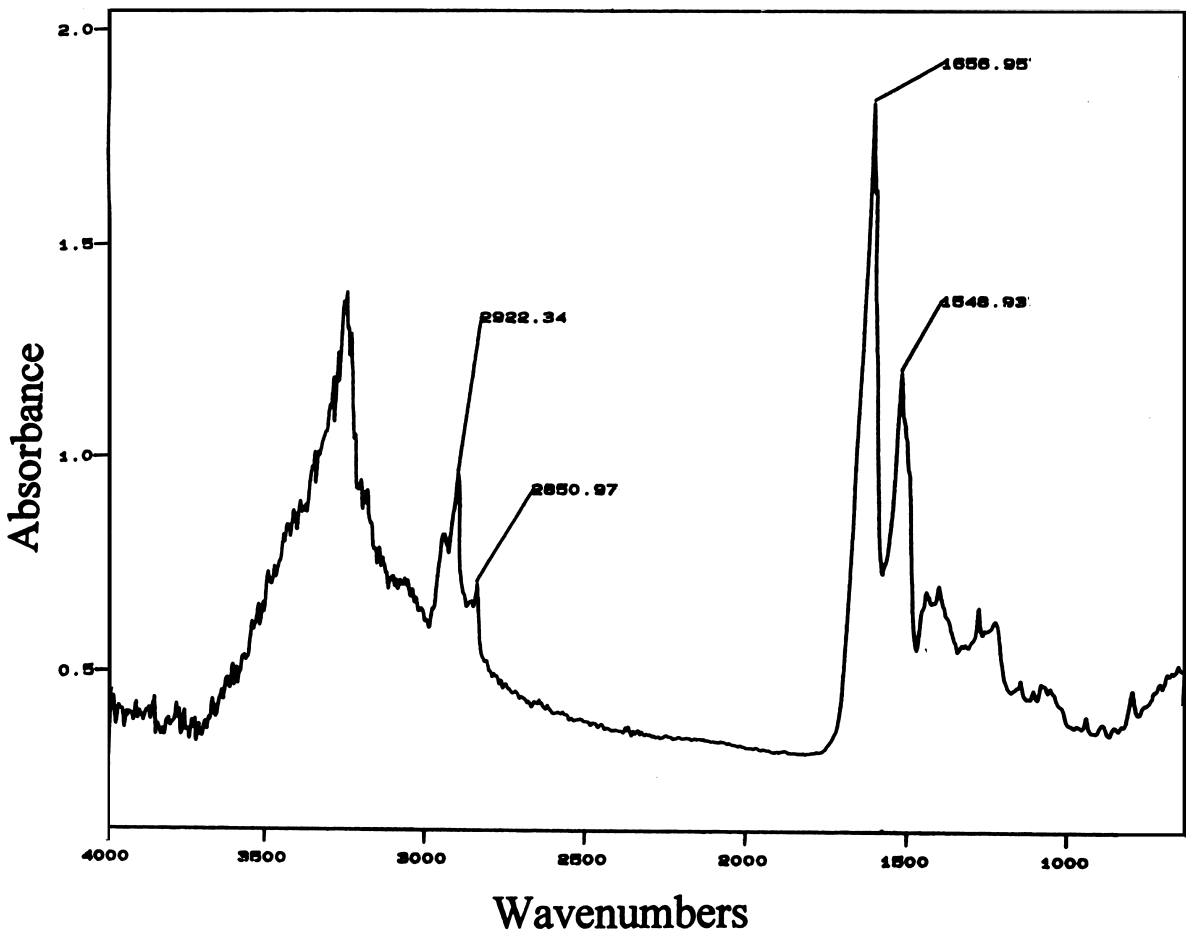

Fig. 5. FT-IR spectrum of porcine stratum corneum treated with 5% thymol in 50% ethanol.

containing terpenes for 5-fluorouracil. Thus, the effect of monocyclic terpenes vary according to physicochemical properties of the drugs. Apparently, hydrocarbon terpenes are effective for lipophilic drugs and oxygen-containing terpenes are effective for hydrophilic drugs. A comparison of 5-fluorouracil flux enhancement by several penetration enhancers can be found in recent publications (Hirvonen et al., 1991; Turunen et al., 1993; Smith and Maibach, 1995).

Terpenes have been found to enhance the permeability coefficient of model hydrophilic 5-fluorouracil (Williams and Barry, 1991a,b) and lipophilic oestradiol (Williams and Barry, 1991a) drugs through the human skin. Williams and Barry (1991b) studied the effect of terpenes on the permeability of 5-fluorouracil through human epidermis, where the effect of two (e.g. carvone and 1,8-cineole) of the three penetration enhancers studied in this work were reported. A comparison of the permeability coefficient and enhancement factor of 5-fluorouracil through different skin types is given in Table 1. The trends in the enhancement of the permeability of 5-fluorouracil due to carvone and 1,8-cineole are similar. 1,8-Cineole produced greater permeability of 5-fluorouracil than the carvone in this study as well as in that of Williams and Barry (1991b). However, the permeability coefficient obtained in this study was greater than in that of Williams and Barry

Table 2
Asymmetric and symmetric C–H stretching IR absorbance peaks of stratum corneum lipids. The results are mean ± S.D. of three determinations

Treatment	C–H stretching absorbance frequency peak (cm^{-1}) (mean ± S.D.)	
	Asymmetric	Symmetric
Control	2917.84 ± 0.91	2850.97 ± 0.00
Carvone	2918.48 ± 0.00	2850.97 ± 0.00
Cineole	2918.48 ± 0.00	2850.97 ± 0.00
Thymol	2922.34 ± 1.57	2850.97 ± 0.00
Water	2918.14 ± 1.82	2850.97 ± 0.00

Control is 50% ethanol in water.

(1991b) treated fully hydrated epidermis with 5-fluorouracil, followed by treatment of the same epidermis with the terpenes, then treatment with 5-fluorouracil again. In this study, we investigated the permeation of 5-fluorouracil in combination with 5% terpene in 50% ethanol. Thus, the greater permeability coefficient in this work may be due to the difference of skin types (porcine skin versus human skin), mode of application and composition of penetration enhancers.

The FT-IR spectrum of the porcine stratum corneum treated with 50% ethanol is shown in Fig. 4. The spectrum has strong amide and water absorbances in the regions of 1500–1700 and 3000–3600 cm^{-1}, respectively. The peaks near 2850 and 2918 cm^{-1} are due to symmetric and asymmetric carbon–hydrogen (C–H) stretching, respectively. Treatment of the stratum corneum with 50% ethanol did not produce a blue shift in the C–H stretching peak positions in comparison to water. However, treatment of the stratum corneum with thymol produced a blue shift in the asymmetric C–H stretching absorbance peak position to higher wavenumbers (Fig. 5 and Table 2). However, carvone or 1,8-cineole did not produce a blue shift in the C–H stretching peak positions. The shift of C–H symmetric stretching peak to higher frequency results when methylene groups along the alkyl chain adopt an increased number of gauche (non-linear) conformers. The molecular basis for the shift in the C–H stretching frequency is well described by Casal and Mantsch (1984).

Table 3
In vitro transepidermal water loss (TEWL) through the epidermis. The results are given as mean ± S.D. of three determinations

Treatment	TEWL (g/m^2 per h) (mean ± S.D.)	TEWL (g/m^2 per h) (mean ± S.D.)[a]
Control	8.30 ± 0.98	
Carvone	15.77 ± 0.56 $P<0.01$, t-test	7.47 ± 0.48
Cineole	15.57 ± 0.40 $P<0.01$, t-test	7.27 ± 1.36
Thymol	22.77 ± 0.95 $P<0.01$, t-test	14.4 ± 0.17

Control is 50% ethanol in water;
[a] TEWL = TEWL through penetration enhancer-treated epidermis − TEWL through the control epidermis.

Fifty percent ethanol in water was used as control to study the transport of 5-fluorouracil. We also investigated the effect of 50% ethanol on the permeability of 5-fluorouracil with respect to water alone. The permeability coefficient of 5-fluorouracil was greater ($P < 0.05$) with 50% ethanol in water [$(0.80 \pm 0.01) \times 10^{-3}$ cm/h] than water alone [$(0.21 \pm 0.05) \times 10^{-3}$ cm/h]. Ethanol is known to enhance the permeation of polar (Kai et al., 1990) and non-polar (Friend et al., 1988) solutes through skin. Kai et al. (1990) found that ethanol enhanced the permeation of polar solute by lipid extraction and not by 'fluidization' of stratum corneum lipid domains. Ghanem et al. (1987) proposed that highly polar molecules permeated through pores in the stratum corneum and found an increase in the permeation of polar molecules through hairless mouse stratum corneum with 50% ethanol due to formation of new pores. Golden et al. (1987b) did not observe alkyl chain fluidization of the stratum corneum lipids by ethanol. In this study also, 50% ethanol did not change the IR spectra in comparison to water. However, there was a slight increase in the in vitro TEWL through 50% ethanol-treated epidermis (8.30 ± 0.98 g/m^2 per h) than water (7.17 ± 0.54 g/m^2 per h), possibly due to formation of new pores.

TEWL results are given in Table 3. Treatments of the epidermis with terpenes (carvone, 1,8-cineole and thymol) have significantly enhanced the in

vitro TEWL ($P < 0.01$) in comparison to the control. TEWL measurements are regarded as an indicator of barrier function, such that a high TEWL generally indicates barrier perturbation.

Several investigators (Wertz and Downing, 1982; Elias, 1983) have suggested that the high resistance of the stratum corneum to water flux is due to the extended multilamellar lipid domains present intercellularly in the stratum corneum. According to this hypothesis, water molecules must traverse the hydrocarbon regions of these lamellae in order to diffuse across this barrier. The diffusion of water molecules through hydrocarbon domains has been measured in a variety of lipid bilayers and liposomes (Boehler et al., 1978; Worman et al., 1986), yielding activation energies for water flux similar to the values reported through the stratum corneum (Golden et al., 1987a). It was suggested that water flux through the stratum corneum was limited by diffusion through the ordered hydrocarbon domains of the intercellular lipids. The study also showed that an increase in the stratum corneum temperature up to about 70°C increased thermal motion of the lipid hydrocarbon chains, in turn allowing greater water flux across these domains. At temperatures above about 70°C, stratum corneum lipids appeared to undergo a phase transition which rendered the stratum corneum lipids fluid and highly permeable to water. It implies that an increase in fluidity of the stratum corneum lipids will result in enhancement of solute flux. Therefore, an increase in the stratum corneum lipids fluidity by enhancers at normal skin temperature (32°C) should increase the permeability of 5-fluorouracil. Our FT-IR findings suggest that thymol increases the degree of disorder of the lipid acyl chains. Thus, an increase in the percutaneous absorption of 5-fluorouracil is highly correlated with an increase in the stratum corneum lipids fluidity. However, carvone or 1,8-cineole did not increase the lipid fluidity but increased the in vitro TEWL. TEWL can be considered a determinant indicative of the functional state of the cutaneous barrier (Wilson and Maibach, 1982; Maibach et al., 1984; Rougier et al., 1989), such that a high TEWL generally indicates barrier perturbation (Pinnagoda et al., 1990). TEWL is widely used to characterize the water barrier function of skin, both in physiological and pathological conditions to perform predictive irritancy tests, and to evaluate the efficacy of therapeutic treatments (Distante and Berardesca, 1995). Rougier et al. (1989) observed a linear relationship between the transepidermal water loss and the percutaneous absorption of molecules. Measurement of TEWL was found to be a relevant parameter for the prediction of percutaneous penetration of water-soluble substances (Lotte et al., 1987). Thus, an increase in the percutaneous absorption of 5-fluorouracil by carvone and 1,8-cineole is highly correlated with the increased in vitro TEWL.

In conclusion, this study has provided information regarding the mechanism of percutaneous penetration enhancement by the test enhancers. Thymol enhanced the percutaneous absorption of 5-fluorouracil by increasing the stratum corneum lipids fluidity and perturbing the barrier integrity of the epidermis. However, the other two terpenes (e.g. carvone and 1,8-cineole) enhanced the percutaneous absorption by perturbing the barrier integrity of the epidermis.

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