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Effects of terpenes and oleic acid as skin penetration enhancers towards 5-fluorouracil as assessed with time; permeation, partitioning and differential scanning calorimetry ☆

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Abstract

Permeation of the model hydrophilic compound 5-fluorouracil was studied through human epidermal membranes treated with penetration enhancers oleic acid in propylene glycol, d-limonene, 1,8-cineole, menthone and nerolidol for times ranging from 1 to 12 h. Partitioning of the drug from aqueous solutions into enhancer treated human stratum corneum was also measured. Differential scanning calorimetry probed the mechanisms of action of these enhancers. All five substances increased the permeation of 5-fluorouracil, probably mainly by disrupting the intercellular lipid lamellar structure. The enhancement effects of d-limonene and oleic acid were saturable within 6 h, reaching a limiting value of about 3.6- and 24-fold increase in drug flux, respectively, whereas 1,8-cineole, menthone and nerolidol showed increasing effects with time leading to maximum enhancements of about 95-, 42- and 25-fold increase, respectively, after 12 h, probably related to improved drug partitioning. Lipid extraction was negligible.

Keywords: Permeation; DSC; Penetration enhancer; Human stratum corneum; 5-Fluorouracil; Terpene; Oleic acid; Propylene glycol

1. Introduction

Skin offers an advantageous route for administering drugs, but its usage is hindered by the poor permeability of the tissue, arising usually from its outermost layer the stratum corneum (Blank, 1965). Human stratum corneum is a hetrogeneous structure consisting of protein-filled corneocytes embedded in multilamellar lipid bilayers. For drug molecules to traverse this layer they may pass through the continuous intercellular lipids or via a transcellular pathway where they have to cross sequentially and repeatedly through the intracellullar protein and the intercellular lipid domains. For most permeants, except the

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most lipophilic ones, the intercellular lipids provide the rate determining component in the skin barrier function.

Many strategies have been suggested to overcome skin impermeability, such as iontophoresis to enhance the transport of peptides or protein drugs (e.g., Chein and Banga, 1989), or the application of supersaturated drug systems (e.g., Davis and Hadgraft, 1991). A popular technique is the use of penetration enhancers which reduce reversibly the permeability barrier of the stratum corneum (Barry, 1983). Over the last two decades much research has concentrated on studying 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 intercellular lipids or the intracellular proteins, or both (e.g., Goodman and Barry, 1988), and/or increasing the partitioning of the drug into stratum corneum (e.g., 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 (e.g.,Ongpipattanakul et al., 1991; Walker and Hadgraft, 1991).

Recently, terpenes, naturally occurring substances, have been investigated as skin penetration enhancers. A series of cyclic monoterpenes were evaluated with 5-fluorouracil (Williams and Barry, 1989, 1991), with indomethacin (Okabe et al., 1989) and with propranolol and diazepam (Hori et al., 1991). 1,8-Cineole, menthone and d-limonene (cyclic monoterpenes), nerolidol (a sesquiterpene) and oleic acid (*cis* unsaturated fatty acid) are among the substances that have been evaluated in our laboratories as skin penetration enhancers towards a model polar drug 5-fluorouracil (Goodman and Barry, 1988; Williams and Barry, 1991; Cornwell and Barry, 1994). The chemical structures of these enhancers are illustrated in Fig. 1.

In our previous studies a single 12 h pretreatment with the test enhancers was employed. The present study probes the mechanisms of action of these skin penetration enhancers, by investigating

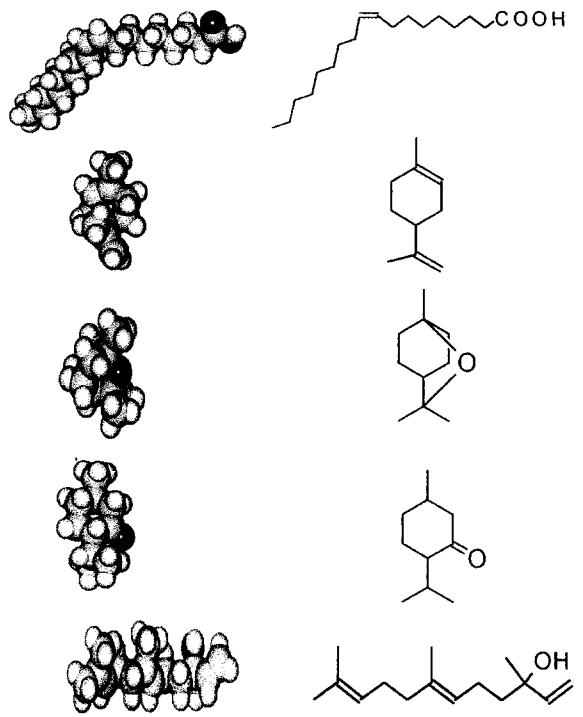

Fig. 1. Molecular structures and space filling (three-dimensional) models of the enhancers (from top to bottom) oleic acid, d-limonene, 1,8-cineole, menthone and nerolidol. Space filling models were produced using the Hyperchem computational chemistry approach. The option used for minimising the charge distribution was the AM1 semi-empirical method; geometry optimization employed the MM+ approach.

the effects of varying the pretreatment time with these materials on the in vitro permeability of 5-flourouracil and on the partitioning of the drug into stratum corneum, and follows the interactions of these enhancers with stratum corneum constituents using differential scanning calorimetry (DSC).

2. Materials and methods

2.1. Materials

1,8-Cineole, d-limonene, oleic acid and propylene glycol were obtained from Sigma Chemical Co. (Poole, UK), and 5-fluorouracil, menthone and nerolidol from Aldrich Chemical Co. (Poole, UK). Tritium-labelled 5-fluorouracil (5-[6-^{3}H]flu-

orouracil) was purchased from NEN (Dupont) Research Products (Germany).

2.2. In vitro permeation studies

Human abdominal skin samples were obtained post-mortem from 24 donors, 73% of whom were female and had a mean age of 71 ± 15 (S.D.) years. Epidermal membranes were prepared using a heat-separation technique (Kligman and Christophers, 1963), by immersing full thickness skin samples in water at 60°C for 45 s. The epidermal membranes were teased off the underlying dermis, and floated on aqueous 0.002% w/v sodium azide solution for 3 days to ensure essentially full hydration of the stratum corneum.

Hydrated epidermal membrane samples were mounted into 0.4 cm diameter stainless-steel diffusion cells of the flow-through design (Akhter et al., 1984). The cells were held on heated metal arms thermally equilibrated at 32 ± 1°C. The receptor fluid was aqueous 0.002% w/v sodium azide, which flowed through the receptor chambers at a rate of 2 ml h^{-1} and hence maintained sink conditions for permeation. Skin samples were pretreated with the test enhancers for times ranging from 1 to 12 h. All enhancers were applied as neat liquids except oleic acid which has been reported to be effective in increasing 5-fluorouracil absorption when applied at 5% w/w in propylene glycol. The permeation of the polar radiolabelled drug 5-fluorouracil from saturated aqueous solutions was assessed before and after enhancer treatment and hence each skin sample acted as its own control. In both the control and the post-treatment runs, the drug transport was assessed for about 36 h using liquid scintillation counting (Tri Carb liquid scintillation analyzer model 1600 TR). The pseudo-steady state permeability coefficients K_p (cm h^{-1}) were calculated from the steady state flux J (cpm cm^{-2} h^{-1}) and the donor concentration C (cpm cm^{-3}) using the relationship:

$$K_p = J/C$$

Enhancer activities were expressed as enhancement ratios, which is the ratio of the drug permeability coefficient after enhancer treatment to that before enhancer treatment. The values reported for each enhancer are the mean from four to six replicates at each pretreatment time.

2.3. DSC

DSC studies used a Perkin Elmer 7 Series thermal analysis system (USA), and skin samples obtained post-mortem from 22 donors 41% of whom were female and had a mean age of 68 ± 14 (S.D.) years. Human stratum corneum membranes were prepared by floating epidermal membranes on 0.0001% w/v trypsin solution containing 0.5% w/v sodium hydrogen carbonate for 12 h. The digested epidermal cells were washed off the stratum corneum sheets with water, and the membranes were then dried, rinsed with ice-cold acetone for 10 s and stored under vaccum over silica gel.

Dry, acetone-rinsed, and weighed (8–12 mg) stratum corneum samples were hydrated over a saturated sodium sulphate solution (R.H. 97% at 25°C) to 20–40% hydration (% hydration = [(wet weight − dry weight)/dry weight] × 100). The stratum corneum samples were reweighed, untreated (to act as controls) or immersed in the test enhancers for 1, 6 or 12 h before they were blotted clean of the excess enhancers and hermetically encapsulated in stainless-steel pans and then analysed from 10 to 140°C at 10°C/min.

The thermograms show endothermic transitions. Due to poor baseline resolution typical of skin, transition midpoint temperatures were determined manually and transition enthalpies were obtained from integration of partial areas. Enthalpies (ΔH, J/g) were calculated from

$$\Delta H = \text{peak area}/\text{sample weight}$$

The results are presented as transition mid point temperatures and their shifts (calculated by substracting control transition temperatures from enhancer treated transition tempratures, i.e., a decrease in transition temperature will produce negtative peak shift), and as enthalpies and enthalpy ratios (calculated by dividing post-treatment enthalpies by control enthalpies, i.e., a decrease in enthalpy will produce ratios less than 1). The data shown are means from three replicates.

Table 1
Permeability coefficients of 5-fluorouracil through human epidermal membranes in vitro, assessed before (K_p) and after ($K_{p,t}$) enhancer treatment

Treatment time (h)	Oleic acid/PG		d-Limonene		1,8-Cineole		Menthone		Nerolidol	
	K_p (cm h^{-1}) ($\times 10^5$)	$K_{p,t}$ (cm h^{-1}) ($\times 10^5$)	K_p (cm h^{-1}) ($\times 10^5$)	$K_{p,t}$ (cm h^{-1}) ($\times 10^5$)	K_p (cm h^{-1}) ($\times 10^5$)	$K_{p,t}$ (cm h^{-1}) ($\times 10^5$)	K_p (cm h^{-1}) ($\times 10^5$)	$K_{p,t}$ (cm h^{-1}) ($\times 10^5$)	K_p (cm h^{-1}) ($\times 10^5$)	$K_{p,t}$ (cm h^{-1}) ($\times 10^5$)
1	3.08 ± 1.16	6.63 ± 1.92	1.66 ± 0.69	2.65 ± 0.98	2.34 ± 0.51	4.49 ± 1.19	0.84 ± 0.25	4.09 ± 1.86	2.19 ± 0.49	3.40 ± 0.77
2	4.35 ± 1.65	15.1 ± 8.80	4.29 ± 1.61	8.99 ± 3.60	3.18 ± 0.85	9.93 ± 3.75	1.73 ± 0.42	6.56 ± 1.66	1.05 ± 0.41	4.09 ± 0.85
4	3.76 ± 0.62	50.4 ± 7.60	1.57 ± 0.54	3.34 ± 1.07	2.18 ± 1.05	19.0 ± 0.93	3.03 ± 0.68	20.9 ± 2.00	1.35 ± 0.01	4.75 ± 0.64
6	5.80 ± 0.49	124 ± 25.7	5.20 ± 1.35	20.4 ± 8.28	5.20 ± 0.4	57.8 ± 9.90	3.13 ± 0.81	32.1 ± 9.80	4.41 ± 0.12	3.87 ± 0.87
8	2.53 ± 0.85	46.5 ± 10.0	3.16 ± 0.98	8.59 ± 3.17	2.67 ± 0.65	53.3 ± 11.8	3.90 ± 0.94	54.5 ± 13.3	0.59 ± 0.13	7.71 ± 2.40
10	2.77 ± 1.03	42.3 ± 16.5	4.39 ± 1.21	16.6 ± 6.81	2.26 ± 0.64	131 ± 40.5	3.13 ± 1.21	177 ± 67.7	2.38 ± 0.37	40.7 ± 3.98
12	2.58 ± 0.63	58.4 ± 12.3	3.23 ± 1.54	8.37 ± 2.28	5.04 ± 1.00	471 ± 87.1	1.42 ± 0.38	53.7 ± 18.5	1.92 ± 0.40	45.7 ± 8.96

Data are summarized as mean ± standard error of mean. $n = 4–6$; PG, propylene glycol.

2.4. Reversibility / lipid extraction studies

Stratum corneum samples were prepared from six donors 33% of whom were female and had a mean age of 75 ± 12 (S.D.). Dry acetone-rinsed stratum corneum samples were weighed, hydrated to 20–40% hydration and treated with the test enhancers, with water as a control or with chloroform/methanol (2:1 v/v) for 12 h. The samples were then removed, blotted free of the excess treating medium and opened flat on wire mesh. The samples were stored under vacuum (650 mmHg) at 21 ± 1°C for 3 days to evaporate off the treatment liquids. The samples were then weighed, sealed in stainless-steel DSC pans and heated from 10 to 140°C at 10°C/min. The results were analysed as in section 2.3. The experiment was carried out in triplicate and enhancer-treated samples were compared with controls from the same sample.

2.5. Partitioning studies

Partitioning studies used skin samples from eight donors 75% of whom were female and had a mean age of 78 ± 10 (S.D.) years. Dry, acetone-rinsed stratum corneum discs were weighed (2–5 mg) and hydrated by floating on 0.002% w/v sodium azide solution for 3 days. The stratum corneum samples were soaked in water (to act as control) or in the test enhancers for 1, 6 or 12 h before incubating them in a subsaturated radiolabelled aqueous solution of 5-fluorouracil at 32 ± 1°C for 24 h. Each stratum corneum sample was then removed from the drug solution, blotted dry, weighed and solubilized using 1 ml of Soluene-350 tissue solubilizer supplied by Packard (Meriden, USA).

The concentrations of 5-fluorouracil in the membranes and in the bathing solutions were determined (in triplicate for the latter) by liquid scintillation counting, and partition ratios were calculated by dividing the stratum corneum/water partition coefficient of the enhancer treated samples by those of the control (untreated) stratum corneum samples. Values reported are mean ratios from three replicates.

3. Results and discussion

The mean permeability coefficient of 5-fluorouracil through untreated human epidermal membranes observed in this study was $3.06 \pm 0.08 \times 10^{-5}$ cm h^{-1} (mean ± SE, $n = 165$) showing good agreement with published values (e.g., 3.50×10^{-5} cm h^{-1} reported by Cohen and Stoughton, 1974; and 2.46×10^{-5} cm h^{-1} reported by Williams and Barry, 1991). Table 1 shows the mean permeability coefficients of 5-fluorouracil before and after treatment of the membranes with oleic acid/propylene glycol, d-limonene, 1,8-cineole, menthone and nerolidol, respectively.

The enhancement effects of the test materials are shown in Fig. 2a and b in terms of enhancement ratios which are the ratio of the permeability coefficients after enhancer treatment to those before enhancer treatment. The permeability coefficient of 5-fluorouracil increased with all the test enhancers, and the mean enhancement ratios (mean ± SE, $n = 4–6$) after 12 h pretreatment with oleic acid/propylene glycol, d-limonene, 1,8-cineole, menthone and nerolidol was 23.6 ± 1.00, 3.55 ± 1.09, 95.3 ± 2.90, 41.9 ± 2.89 and 25.3 ± 1.67, respectively, showing good agreement with the mean published data of 16.2, 2.19, 94.5, 37.9 and 22.8, respectively (Goodman and Barry, 1988; Williams and Barry, 1991; Cornwell and Barry, 1994).

Oleic acid and d-limonene (Fig. 2a) gradually increased the enhancement ratios up to pretreatment times of 6 h, after which the enhancement effect reached a plateau. 1,8-Cineole (Fig. 2b) gradually increased the permeation of 5-fluorouracil as the pretreatment time was increased from 1 to 8 h, followed by a sharp increase leading to the maximum enhancement ratio of 95.3 measured after 12 h; while menthone and nerolidol (Fig. 2b) increased the ratios more gradually and continuously. Thus, as the pretreatment time with the test enhancers increased, their enhancement effects divided them into two groups; oleic acid and d-limonene provided saturable effects while the enhancement induced by 1,8-cineole, menthone and nerolidol escalated as the treatment time prolonged.

Saturation of enhancer effects has been re-

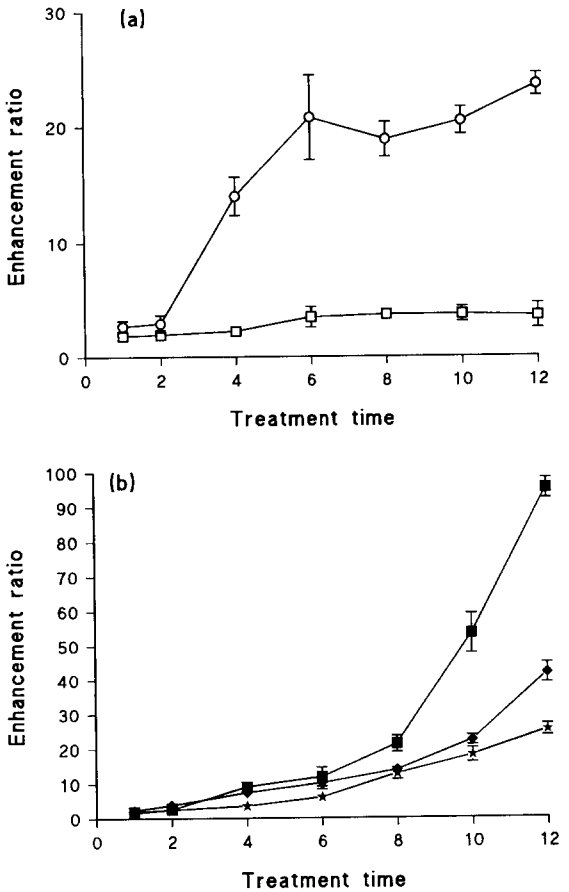

Fig. 2. (a) The effect of the skin penetration enhancers d-limonene (□) and oleic acid 5% w/w in propylene glycol (○) on the permeation of 5-fluorouracil through human epidermal membranes in vitro assessed after different pretreatment times. The data represent the mean and SE for four to six replicates at each point (error bar lines are within the size of symbol where not shown). (b) The effect of the skin penetration enhancers 1,8-cineole (■), menthone (♦) and nerolidol (★) on the permeation of 5-fluorouracil through human epidermal membranes in vitro assessed after different pretreatment times. The data represent the mean and SE for four to six replicates at each point (error bar lines are within the size of symbol where not shown).

ported previously by other workers. For example, propranolol penetration rates across hairless mouse skin reached a maximum enhancement when examined as a function of *n*-nonane and *n*-nonanol concentration in the applied formulation (Hori et al., 1991). The authors presumed that the stratum corneum was maximally perturbed beyond which the penetration rate was controlled by another event unaffected by the enhancers. Azone increased the permeablilty coefficients of butanol, vidarabine and hydrocortisone through hairless mouse skin up to a limiting value in spite of increasing further its applied amount per unit area of stratum corneum (Lambert et al., 1989). In another study (Turunen et al., 1993), the flux of 5-fluorouracil through shed snake skin pretreated with dodecyl *N,N*-dimethylamino isopropionate increased with increasing the pretreatment time from 2 to 6 h after which the flux reached a plateau, while dodecyl *N,N*-dimethylamino acetate provided a saturable effect within 2 h pretreatment time. However, in a study using shed snake skin pretreated with Azone, the penetration of sulfanilamide increased with increasing applied concentrations of Azone; the results led to the proposal of lipid fluidization and the release of some structured lipids from the skin into the donor solution as a mechanism of Azone action (Ogiso et al., 1992).

The calculation of enhancement ratios in our study was based on estimating the permeability coefficient of the drug before and after enhancer treatment. The permeability coefficient is a bi-component factor (assuming that stratum corneum thickness does not change) including both the diffusion coefficient or diffusivity and partition coefficient; the enhancement effect of the accelerants may involve one of these components or both. One likely mechanism involved in increasing drug diffusivity is the disruption of the highly organized intercellular lipid domain. The contribution of this mechanism to the observed increase in drug permeability caused by the test enhancers was studied using DSC.

A thermogram of human stratum corneum (Fig. 3, top thermogram) typically shows four major endothermic transitions; T1 at around 37°C, T2 at around 72°C, T3 at around 83°C and T4 at around 100°C (Van Duzee, 1975). The first three were lost following treatment of stratum corneum samples with lipid-extracting solvents, suggesting that they derive from thermal events within the stratum corneum lipids, while T4 was heat irreversible and remained after lipid extraction, suggesting that it arises from a protein component (Goodman and Barry, 1989).

Table 2
Effects of enhancers on the transition temperatures (°C) of the lipid related thermal transitions (T2 and T3) in human stratum corneum ($n = 3$)

Treatment time (h)	Oleic acid				d-Limonene				1,8-Cineole				Menthone				Nerolidol			
	T2		T3		T2		T3		T2		T3		T2		T3		T2		T3	
	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T
1	72.0	59.1	85.1	69.9	72.4	50.1	83.9	66.6	72.4	49.4	83.9	68.4	71.3	51.5	82.3	70.7	73.2	70.5	84.8	82.3
6	73.5	59.3	83.5	70.8	75.4	50.7	86.9	66.4	75.4	51.1	86.9	70.9	70.8	50.9	81.6	71.5	73.2	69.8	85.1	80.7
12	73.3	58.3	83.6	71.2	71.3	49.2	84.7	65.8	71.3	47.5	84.7	69.8	70.1	50.7	80.4	71.4	73.2	68.9	85.1	81.1

C, transition temperature of the untreated samples; *T*, transition temperature of the treated samples. Values represent means, SE < 3.5%.

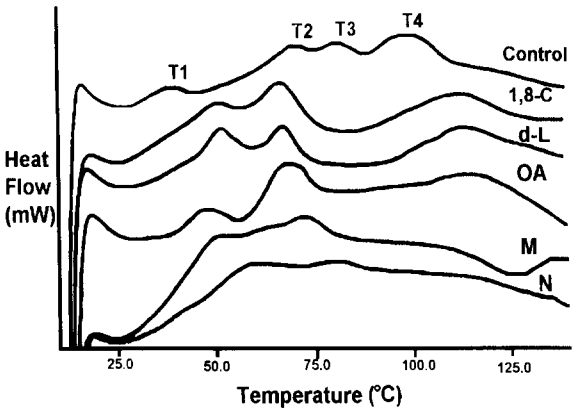

Fig. 3. DSC thermograms showing (from top to bottom) 20–40% hydrated stratum corneum (control) and stratum corneum treated for 12 h with 1,8-cineole (1,8-C), d-limonene (d-L), oleic acid 5% w/w in propylene glycol (OA), menthone (M) and nerolidol (N).

T1 has the lowest enthalpy, and it was absent in some samples. Whilst this endotherm has been attributed to sebaceous secretions or surface contaminations, a recently suggested origin for this feature is that it may arise from minor structural rearrangement within the bilayers (White et al., 1988; Bouwstra et al., 1992). In addition, a recent study has shown that no marked change in in vitro skin permeability towards ions and 5-flourouracil occurred at temperatures above T1 and below T2, suggesting that the lipid barrier function remained essentially unchanged (Cornwell and Barry, 1993). Thus, endotherm T1 is unlikely to be of major importance when investigating the mechanism of action of penetration enhancers.

There is general agreement that endotherm T2 derives from the melting of bilayer lipids, while interpretations of the endotherm T3 are conflicting (as a reference for the different interpretations see Williams and Barry, 1992). More recently, Cornwell et al. (1994a) suggested that non-random distribution of the lipids in the intercellular bilayers led to the melting of stratum corneum lipids in two stages, hence the presence of T2 and T3.

Several studies have tried to relate changes in the endotherms T2 and T3 to changes in in vitro skin permeability. Golden et al. (1987) observed a linear relationship between flux enhancement of salicylic acid through porcine skin and changes in the thermal transition near 65°C for a series of isomers of octadecanoic acid. In our study, all the test enhancers reduced the transition temperatures of the two lipid related endotherms T2 and T3 (Fig. 3 and Table 2), suggesting a disruption in the lipid bilayers. In agreement with previous studies (Williams and Barry, 1989; Cornwell and Barry 1992), the transition temperature shifts were not, however, predictive of potencies of all terpenes. Following 12 h pretreatment, the terpenes provided enhancement ratios of: d-limonene, 3.55; nerolidol, 25.3; menthone, 41.9 and 1,8-cineole, 95.3 while their effects on the transition temperature shifts were: nerolidol shifts T2 −4.2°C and T3 −4°C, menthone shifts T2 −19.4°C and T3 −10.1°C, and 1,8-cineole and d-limonene provided near identical effects (they shift T2 and T3 about −23 and −16°C, respectively). Thus, whilst nerolidol, menthone and 1,8-cineole showed a rank correlation, transition shifts were not predictive of d-limonene potency compared with the other terpenes. Similarly, transition temperature shifts were not predictive in relating the enhancement effect of oleic acid to the terpenes.

Fig. 4a and b shows a comparison between the shifts in the transition tempratures of T2 and T3 produced by each enhancer at different pretreatment times, expressed as minus shift. As can be seen, the endotherm shifts for each enhancer were similar (no significant difference, Tukey's 'HSD test', $\alpha = 0.05$) in spite of increasing the pretreatment time. This behaviour correlates with the permeation results for the enhancers which produced saturable effect (i.e., d-limonene and oleic acid), but does not correlate with the results of the other (non-saturable) group.

More information about the interactions of the enhancers with stratum corneum components was gained by determining the endotherm enthalpies (Table 3). Fig. 5a and b demonstrates the effects of the enhancers on the enthalpies expressed as enthalpy ratios, which is the ratio of the enthalpy of the treated sample to the enthalpy of the control. While 1,8-cineole and d-limonene pro-

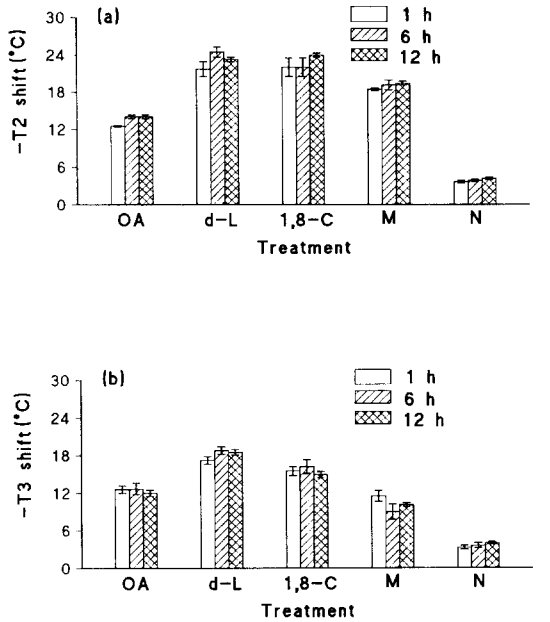

Fig. 4. Histograms showing the effect of pretreatment time with the enhancers on the shifts of T2 (a) and T3 (b). Data represent the mean and SE, $n = 3$. Symbols as for Fig. 3.

duced similar shifts in the transition temperatures of T2 and T3, suggesting that they produced the same degree of disruption in the lipid bilayers, up to 12 h pretreatment with d-limonene produced no marked effect on the enthalpy ratios of T2 and T3. However, 1,8-cineole markedly reduced these ratios by about 50%. This observation correlates with the permeation results, and indicates that enthalpy ratios might be a more predictable measure of enhancers potencies.

Enthalpy ratios were also predictable in assessing the effect of a given enhancer with time. The reduction in the enthalpy ratios of T2 produced by 1,8-cineole became more marked as the pretreatment time was prolonged, which may corresponds to an increase in the fluidization of the lipids. Moreover, a plot of T2 enthalpy ratios versus enhancement ratios yielded a linear correlation, $r = 0.92$.

Oleic acid produced no marked effect on the enthalpy of T3, but it reduced the enthalpy of T2 after 6 h pretreatment by about 50%, which was sustained for a 12 h treatment time, in agreement with its saturable effect on the permeation of 5-fluorouracil. A decrease in the enthalpies associated with the lipid transitions of porcine skin with oleic acid (0.25% v/v in ethanol/water) has been reported (Francoeur et al., 1990), but a similar reduction was observed with ethanol/water controls. In contrast, the reduction in T2 enthalpies in our study was specific to oleic acid, since oleic acid was applied in propylene glycol and the latter has no major effect on lipid transition temperatures or enthalpies (Goodman and Barry, 1989).

Nerolidol and menthone shifted the transition temperatures of T2 and T3 to lower temperatures (Fig. 3 and 4a,b), suggesting a disruption in the lipid bilayers. In addition they broadened the two peaks; this phenomenon normally correlates with a decrease in bilayer cohesion. The broadening of the endotherms provided difficulties in the determination of accurate peak enthalpies. An apparent increase in the enthalpy ratios of the two peaks following nerolidol and menthone treatment was observed (Fig. 5a,b).

The effects of menthone and nerolidol on the lipid transition temperatures and enthalpies appear to be contradictory. Shifting the endotherms

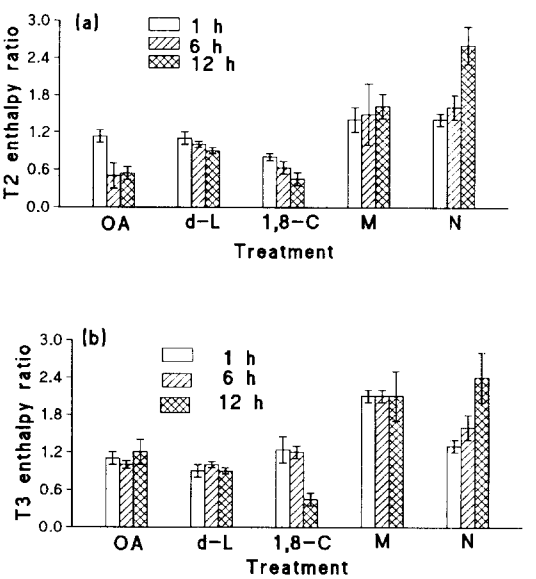

Fig. 5. Histograms showing the effect of pretreatment time with the enhancers on the enthalpies of T2 (a) and T3 (b). Data represent the mean and SE, $n = 3$. Symbols as for Fig. 3.

Table 3
Effects of enhancers on the transition enthalpies (J/g) of the lipid related thermal transitions (T2 and T3) in human stratum corneum ($n = 3$)

Treatment time (h)	Oleic acid				d-Limonene				1,8-Cineole				Menthone				Nerolidol			
	T2		T3		T2		T3		T2		T3		T2		T3		T2		T3	
	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T
1	7.23	7.19	7.35	8.31	7.32	7.30	5.07	4.49	7.32	5.80	5.07	5.91	4.46	7.35	4.94	10.4	5.69	7.85	4.92	6.26
6	8.87	3.53	5.70	5.74	7.60	7.61	5.95	6.40	7.60	5.51	5.95	7.30	5.14	10.2	4.74	9.99	5.72	10.2	4.98	9.01
12	10.1	6.48	7.26	8.34	5.32	4.90	4.53	4.08	5.32	2.43	4.53	2.05	4.76	7.69	4.95	8.24	5.40	13.5	4.75	11.3

C, transition enthalpy of the untreated samples; T, transition enthalpy of the treated samples. Values represent means, SE $\leq 15\%$.

to lower temperatures and broadening them suggests a disruption or fluidization in the lipid bilayers, which corrèlates with their enhancement effect towards 5-fluorouracil permeation, while increasing the transition enthalpies suggests an increase in the bilayers co-operativity. However, in recent studies, small angle X-ray diffraction results have shown that this lipid disruption is achieved without decreasing the bilayers periodicity, indeed some bilayer reflections appeared to be slightly enhanced (Cornwell et al., 1994b), possibly arising from lateral bilayer swelling. This effect may explain the observed apparent increase in the lipid peak enthalpies.

All the test enhancers affected the protein related peak T4 (Fig. 3). 1,8-Cineole and d-limonene shifted the transition temperature of T4 to higher temperatures, and broadened the peak. This effect may be due to tissue dehydration. Oleic acid/propylene glycol removed T4, but this effect is not solely due to oleic acid since pure propylene glycol produces the same effect (Goodman and Barry, 1989), again, probably due to tissue dehydration. T4 was also lost following menthone and nerolidol treatments, either due to the broadening of T3 or due to tissue dehydration. The effects of the test enhancers on the protein transition may also indicate enhancer/protein interactions. Raykar et al. (1988), using a series of hydrocortisone 21-esters, have shown that members of this series with lipophilicities comparable to those of the terpenes were able to partition into the intracellular protein domains as well as into the intercellular lipid region, though less favourably to the former. Thus, the effects of the enhancers on T4 might be due to a direct interaction with the protein and not entirely due to tissue dehydration.

In a parallel experiment, DSC was used to investigate the reversibility of the effects of the enhancers on the lipid endotherms and consequently as an indirect, qualitative (but yet reliable) measure of lipid extraction by the enhancers. It was necessary to investigate this possibility of lipid extraction, especially for the enhancers which produced non-saturable enhancement effects on 5-fluorouracil permeation (1,8-cineole, menthone and nerolidol), since a similar non-saturable enhancement effect of Azone on sulfanilamide permeation through shed snake skin was reported to be (at least in part) due to lipid extraction (Ogiso et al., 1992). On the other hand, the reduction in the enthalpy of the lipid related endotherms by 1,8-cineole and oleic acid may indicate fluidization in the lipid bilayers and may at the same time indicate lipid extraction. Hence, this experiment was used to clarify the involved mechanism.

As detailed in section 2, stratum corneum samples were pretreated with the enhancers, with water as negative control of lipid extraction or with chloroform/methanol as a lipid extracting solvent (positive control). The stratum corneum samples were then removed from the bathing medium and the partitioned liquid was evaporated from stratum corneum samples which were subjected to DSC analysis. It is worth noting that after the process of enhancer or chloroform/methanol treatment and evaporation the samples resisted rehydration to the required level (20–

Table 4
Temperatures and enthalpies of the lipid related endotherms T2 and T3 following the evaporation of the treating substance

Treatment	Transition T2		Transition T3	
	Temperature (°C)	Enthalpy (J/g)	Temperature (°C)	Enthalpy (J/g)
Water	77.4 ± 0.3	12.6 ± 1.3	90.4 ± 1.0	7.78 ± 0.4
Oleic acid	75.0 ± 0.7	13.9 ± 2.4	86.9 ± 0.9	8.69 ± 0.5
d-Limonene	77.1 ± 0.9	14.8 ± 1.2	90.4 ± 1.9	9.25 ± 0.8
1,8-Cineole	76.0 ± 0.9	12.6 ± 1.6	88.0 ± 1.2	7.50 ± 0.6
Menthone	77.1 ± 0.8	14.4 ± 3.5	88.6 ± 1.0	7.62 ± 0.9
Nerolidol	72.9 ± 0.9	15.8 ± 3.6	84.6 ± 0.4	11.8 ± 0.4

Data summarized as mean ± standard error of mean, $n = 3$.

Table 5
Stratum corneum/water partition coefficient of 5-fluorouracil into untreated control (P_c) and enhancer-treated stratum corneum (P_t)

Treatment time (h)	Oleic acid/PG		d-Limonene		1,8-Cineole		Menthone		Nerolidol	
	P_c	P_t	P_c	P_t	P_c	P_t	P_c	P_t	P_c	P_t
1	2.67 ± 0.49	1.29 ± 0.25	2.67 ± 0.49	1.19 ± 0.05	2.67 ± 0.49	1.14 ± 0.03	0.76 ± 0.07	0.90 ± 0.06	0.76 ± 0.07	1.03 ± 0.09
6	1.34 ± 0.13	1.10 ± 0.05	1.34 ± 0.13	1.34 ± 0.06	1.34 ± 0.13	1.85 ± 0.15	0.81 ± 0.14	1.44 ± 0.39	0.81 ± 0.14	1.54 ± 0.08
12	1.43 ± 0.17	1.04 ± 0.16	1.43 ± 0.17	1.24 ± 0.09	1.43 ± 0.17	3.46 ± 0.47	0.93 ± 0.14	2.00 ± 0.41	0.93 ± 0.14	2.20 ± 0.52

Data are summarized as mean ± standard error of mean. $n = 3$; PG, propylene glycol.

40%), which may indicate that some natural moisturising factors were extracted during the process. Hence, the samples were submitted to DSC analysis in essentially the dry state, consequently, the transition midpoint temperatures and enthalpies were higher than those of the hydrated samples.

In stratum corneum samples which were treated with chloroform/methanol, the lipid related peaks were completely absent (after the evaporation of the solvent from the samples) indicating that most of the lipid constituents had been extracted from the the stratum corneum into the bathing chloroform/methanol solution. In contrast (see Table 4), for stratum corneum samples treated with the enhancers, the transition midpoint temperatures and the enthalpies of the endotherms were restored to the initial values. With nerolidol the recovery was not complete, probably due to the incomplete evaporation of the long chain terpene. Fig. 6a and b compares the enthalpy ratios of T2 and T3 in the presence of the enhancers to those after enhancer evaporation. The ratios were very close to unity after the removal of the enhancers. The reversibility in the effect of the enhancers and, on the other hand, the dramatic and unrecoverable effect of chloroform/methanol provide strong evidence that the enhancers did not extract significant amount of lipids from stratum corneum.

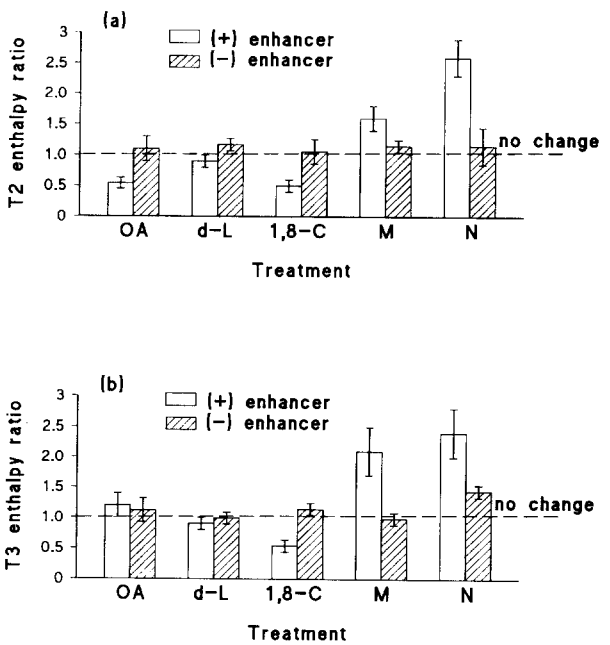

Fig. 6. Histograms comparing the enthalpy ratios of T2 (a) and T3 (b) in the presence of the enhancers to those after enhancer removal. Data represent the mean and SE, $n = 3$. Symbols as for Fig. 3.

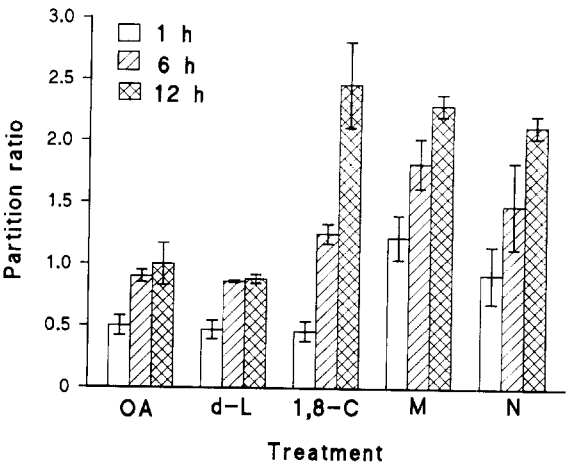

Fig. 7. Histograms showing the effect of pretreatment time with the enhancers on the partitioning of 5-flourouracil into human stratum corneum. Data represent the mean and SE, $n = 3$. Symbols as for Fig. 3.

The mean stratum corneum/water partition coefficient of 5-fluorouracil into essentially fully hydrated untreated stratum corneum samples and into stratum corneum samples treated with the enhancers for different treatment times are given in Table 5. The results are presented in term of partitioning ratios in Fig. 7 which were calculated by dividing the stratum corneum/water partition coefficient of the enhancer treated samples by those of the control (untreated) stratum corneum samples.

Initially, a reduction in the partitioning of the drug after 1 h pretreatment with 1,8-cineole, d-limonene and oleic acid was observed. The pooling of enhancers inside stratum corneum has been proposed as an explanation for the uptake of large quantities of nerolidol, 1,8-cineole and d-limonene into stratum corneum which were reported to be 38.0, 26.3 and 8.90% w/dry weight of stratum corneum (Cornwell et al., 1994a). If

the enhancers replace water in the stratum corneum, we expect this observed initial reduction in drug partitioning into stratum corneum treated with 1,8-cineole and d-limonene, since 5-fluorouracil is relatively less soluble in 1,8-cineole and d-limonene (0.249 and 0.0057 mg/ml, respectively) than water (14.3 mg/ml). However, nerolidol (5-fluorouracil solubility in nerolidol is 0.46 mg/ml) did not cause this initial reduction and hence the decrease in drug partitioning is unlikely to be due to a simple solvent effect. Schuckler and Lee (1992) have reported a similar initial decrease in the partition coefficient of diazepam at low loadings with Azone followed by no clear dependence on enhancer concentration therafter. However, dodecyl pyroglutamate provided an initial decrease in diazepam partition coefficient followed by a sudden increase after approx. 12% loading.

As the treatment time was increased to 6 and 12 h, d-limonene and oleic acid produced no positive effect on the partitioning of the drug, while 1,8-cineole, menthone and nerolidol induced an increase. It should be noted that we have employed aqueous drug solutions and used enhancer pretreated stratum corneum, and hence any observed change in the partitioning of the drug is essentially due to a change in the stratum corneum. 1,8-Cineole, menthone and nerolidol increased drug partitioning by changing the solubility properties of the tissue. They may dissolve to some extent in the rate determining site in the stratum corneum domain, thus increasing 5-fluorouracil solubility in this region. Such a phenomenon of increasing skin partitioning of a polar drug following pretreatment with non-polar enhancers has been reported previously. For example, Okamoto et al. (1988) reported an increase in the partitioning of 6-mercaptopurine following pretreatment with Azone. Similarly, Sasaki et al. (1991) revealed an increase in skin accumulation of 5-fluorouracil in the presence of alkyl pyrollidones.

The enhancement ratios of the accelerants can thus be related to their effects on the lipid endotherms (as an indirect measure of the effect on diffusivity) and to their modifications of drug partitioning. 1,8-Cineole provided the highest enhancement effect, shifted lipid derived endotherms to lower temperatures, reduced their enthalpies and increased drug partitioning. Menthone and nerolidol provided an intermediate enhancement effect; they moved the endotherms to lower temperatures, produced an apparent increase in enthalpies and improved drug partitioning. Oleic acid also provided an intermediate enhancement effect; it shifted the endotherms to lower temperatures and reduced the enthalpy of T2 without improving drug partitioning. d-Limonene provided the mildest enhancement effect; it shifted the endotherms to lower temperatures without affecting the enthalpies or drug partitioning.

In conclusion, this study has provided information regarding enhancer skin interactions as assessed with time. Permeation and DSC studies have indicated that the mechanism of enhancement of all the test accelerants may involve increasing drug diffusivity, probably by disrupting the intercellular lipid domain. Combining the permeation studies with partitioning studies has shown that the enhancement effect was saturable with the enhancers which were unable to improve the partitioning of the drug (d-limonene and oleic acid), while the improvement of drug partitioning with 1,8-cineole, menthone and nerolidol contributed to the overall increase in drug absorption with these enhancers. Lipid extraction was unimportant.

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