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Sesquiterpene Components of Volatile Oils as Skin Penetration Enhancers for the Hydrophilic Permeant 5-Fluorouracil

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Abstract—Twelve sesquiterpene compounds, derived from natural volatile oils, were investigated as putative skin penetration enhancers for human skin. Pretreatment of epidermal membranes with sesquiterpene oils, or solid sesquiterpenes saturated in dimethyl isosorbide, increased the rate of absorption of the model hydrophilic permeant, 5-fluorouracil (5-FU). Enhancers with polar functional groups were generally more potent than pure hydrocarbons. Furthermore, enhancers with the least bunched structures were the most active. The largest effect was observed following pretreatment with nerolidol, which increased pseudo-steady-state 5-FU flux over 20-fold. Molecular modelling suggested that terpenes with structures suitable for alignment within lipid lamellae were the most potent enhancers. Sesquiterpene enhancers had long durations of action implying that they did not wash out of the skin easily. This study attempted to improve enhancer clearance by replacing the aqueous donor and receptor phases by ethanol:water (1:1) solutions. Ethanol increased the permeability coefficient for 5-FU 13-fold, demonstrating that, in aqueous solution, it is a moderately potent penetration enhancer. Sesquiterpene and ethanol enhancement effects were approximately additive. Sesquiterpene effects were almost fully maintained for at least 4·5 days following pretreatment, illustrating poor reversibility. Stratum corneum/water drug partitioning studies suggested that an important mechanism of action of the enhancers was to increase the apparent drug diffusivity in the stratum corneum. Increases in drug partitioning into the entire stratum corneum following enhancer pretreatment were relatively small. Diffusivity increases were directly related to overall rises in permeability. This study has shown that sesquiterpene compounds, which are of low toxicity and cutaneous irritancy, can promote 5-FU absorption across human skin. Sesquiterpene compounds, therefore, show promise as clinically-acceptable skin penetration enhancers.

Percutaneous drug delivery via transdermal devices offers the possibility of providing sustained drug plasma levels and of avoiding hepatic first-pass metabolism. In addition, patches improve patient compliance with long-term treatment regimens and allow termination of therapy by the patient simply removing the device from the skin (assuming negligible drug reservoir exists in the skin). Unfortunately, many drugs and bioactive peptides cannot penetrate undamaged skin in therapeutic quantities, posing problems for formulators. A popular method advanced for improving drug delivery is to employ skin penetration enhancers (Williams & Barry 1992). Natural volatile oils are commonly of low cutaneous irritancy and are therefore good candidates for useful skin penetration enhancers. To date, most investigations have focused on the monoterpene constituents of essential oils. Monoterpenes have been shown to be effective penetration enhancers for both hydrophilic drugs (Williams & Barry 1990; Takayama et al 1990; Hori et al 1991) and lipophilic drugs (Okabe et al 1989; Hori et al 1991; Williams & Barry 1991a).

The present study evaluates the penetration enhancing abilities of further natural volatile oil constituents, the sesquiterpenes. Sesquiterpenes are synthesized from three isoprene units, and are isolated from the higher boiling point fractions of commonly used essential oils. Cedrene, for example, is a constituent of cedarwood oil, and farnesol a component of rose oil. In this study twelve sesquiterpene compounds are investigated as penetration enhancers for the model hydrophilic drug, 5-fluorouracil (5-FU). The compounds selected are generally of low toxicity and cutaneous irritancy (Table 1) and are chosen to represent the major structural classes of sesquiterpenes commercially available in a purified form (Fig. 1). Fig. 2 illustrates space-filling models of the terpenes developed using the HyperChem computational chemistry approach. The option used for minimizing the charge distribution was the AM1 semi-empirical method; geometry optimization employed the MM^+ approach.

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Materials and Methods

Materials

5-[6^{3}H]Fluorouracil was obtained from NEN (Dupont) Research Products (Dreiech, Germany). Radiochemical purity was tested using thin-layer chromatography. On a silica gel G (250 μm) plate and with an ethyl acetate:methanol (3:1) mobile phase, 5-FU separated with an R_f value of approximately 0·6. 5-[6^{3}H]Fluorouracil was determined to be 98·0% pure.

The sesquiterpenes (+)-longifolene, (+)-aromadendrene, (+)-β-cedrene, (−)-guaiol, (+)-cedrol and (+)-cedryl acetate were obtained from Fluka (Buchs, Switzerland). β-Caryophyllene oxide, nerolidol (mixture of isomers) and farnesol (mixture of isomers) were purchased from Aldrich (Gillingham, UK). *Trans*-β-caryophyllene was purchased from Sigma Chemical Co. (St Louis, MO). (−)-α-Bisabolol was received as a gift from BASF (Cheadle, UK). Compound purities were tested by capillary gas chromatography (GC). GC studies used a 25 m BP-5 capillary column (SGE Inc.,

Table 1. Sources, purities, melting points and toxicological data of sesquiterpenes.

Compound	Example of a major source	Compound purity[a] (%)	Melting point[b] (°C)	Acute oral LD50	Skin irritancy[c]	Toxicity data monograph
(+)-Longifolene	Turpentine oil	100	Liquid	—	—	—
β-Caryophyllene	Clove oil	100	Liquid	>5 g kg^{-1}[d]	None (4% in petrolatum)	Opdyke (1973)
(+)-Aromadendrene	Eucalyptus oil	100	Liquid	—	—	—
(+)-β-Cedrene	Cedarwood oil	100	Liquid	>5 g kg^{-1}[d]	None (5% in petrolatum)	Opdyke (1978)
(−)-Isolongifolol	Synthetic derivative	100	113–114	—	—	—
(−)-Guaiol	Guaiacwood oil	98·6	91–93	—	—	—
(+)-Cedrol	Cedarwood oil	87·3	82–86	>5 g kg^{-1}[e]	None (8% in petrolatum)	Opdyke (1975a)
(−)-α-Bisabolol	Camomile oil	97·1	Liquid	>5 g kg^{-1}[d]	—	BASF data
Farnesol	Rose oil	97·2	Liquid	—	—	—
Nerolidol	Cabreuva oil	100	Liquid	>5 g kg^{-1}[d]	None (5% in petrolatum)	Opdyke (1975b)
β-Caryophyllene oxide	Synthetic derivative	95·9	61–62	>5 g kg^{-1}[d]	None (4% in petrolatum)	Opdyke (1983)
(+)-Cedryl acetate	Synthetic derivative	83·0	44–46	—	—	—

[a] Determined by capillary gas chromatography. [b] Manufacturer's data. [c] Forty-eight-hour closed patch test in man. [d] Acute oral LD50 in rat. [e] Acute oral LD50 in rabbit.

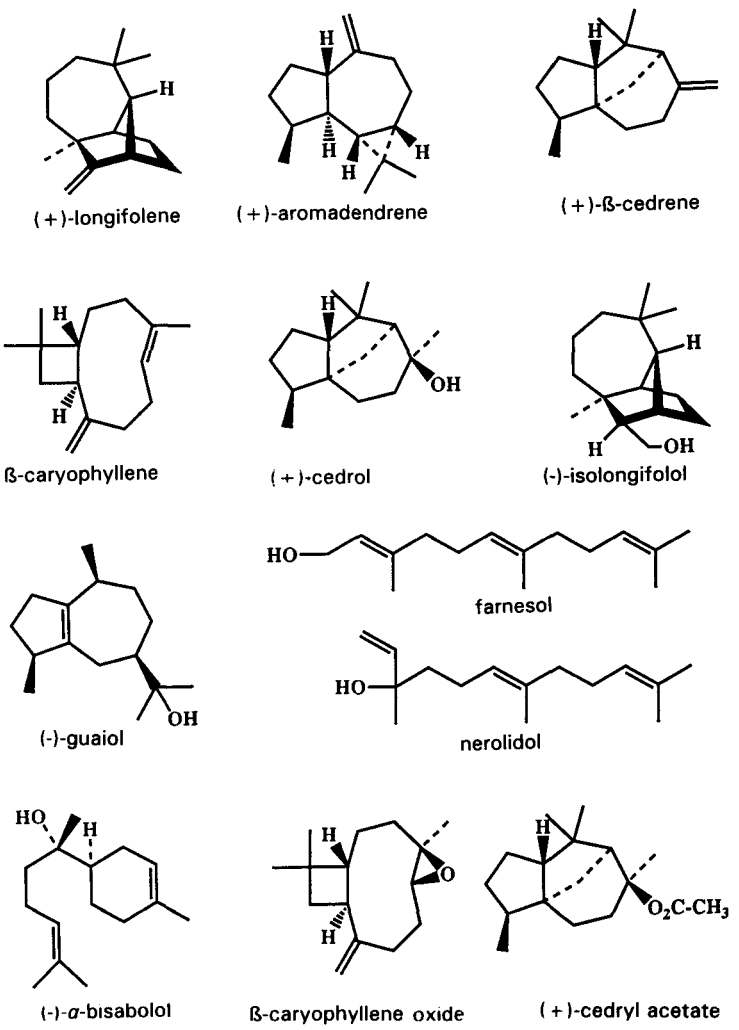

FIG. 1. Molecular structures of the sesquiterpene compounds used.

Australia) and helium (8 psi) as the carrier gas. The oven temperature was raised from 50°C at injection to 275°C at a rate of 2°C min^{-1}. The eluate was analysed by a hydrogen-flame ionization detector maintained at 285°C. Integrated peak areas were used for purity determinations. Table 1 summarizes the determined purities. Most compounds were >97% pure, except β-caryophyllene oxide (95·9%), (+)-cedrol (87·3%) and (+)-cedryl acetate (83·0%). The major impurity present in (+)-cedrol and (+)-cedryl acetate was identified as cedrene. Fortunately, cedrene is a weak penetration enhancer for 5-FU (Table 2) and thus is unlikely to markedly affect the activities of the major constituents.

Dimethyl isosorbide was purchased from Aldrich. All other solvents and reagents were of analytical grade.

Epidermal membranes

Human abdominal cadaver skin was obtained post-mortem and stored frozen at −20°C in double-sealed evacuated polythene bags (Harrison et al 1984). Epidermal membranes were prepared by immersing full thickness skin samples,

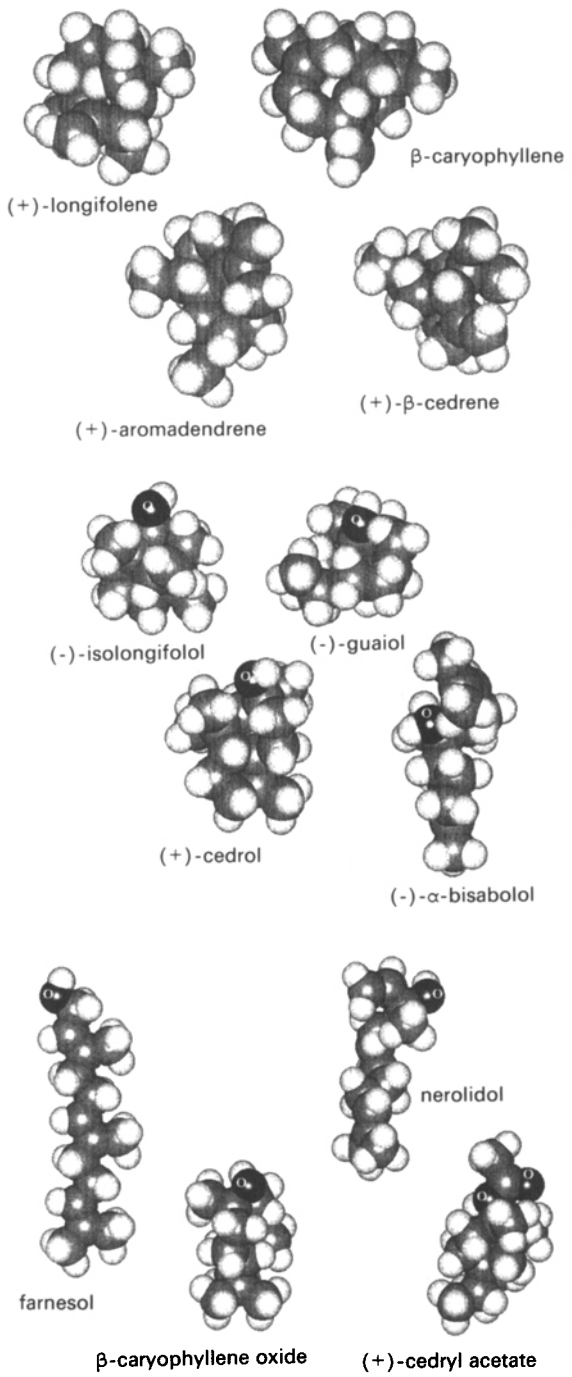

FIG. 2. Space-filling (3D) models of the sesquiterpenes; oxygen molecules marked O.

trimmed of subcutaneous fat, in water at 60°C for 45 s; the epidermal membranes could then be gently peeled off the underlying dermis (Kligman & Christophers 1963). Hairy skin samples tended to create tears in the membranes and were thus avoided. Epidermal membranes from 19 different donors were used for the diffusion studies. Donors were predominantly elderly (aged 66·5 ± s.d.17·7 years) and female (82%).

Stratum corneum

Stratum corneum sheets were prepared by floating epidermal membranes on an aqueous solution of 0·0001% trypsin (Sigma) and 0·5% sodium bicarbonate for 12 h. Digested material was removed from the underside of the stratum corneum with tissue paper and the isolated sheets were rinsed in an aqueous solution of 0·002% sodium azide.

Cleaned sheets were dried on PTFE-coated wire meshes under ambient conditions. Each sheet was rinsed in acetone for 20 s, removing any sebaceous or subcutaneous fat contamination, and stored for up to two weeks over silica gel, under vacuum.

Partitioning studies used stratum corneum isolated from 12 different skin samples. Donors, like those for the diffusion studies, were elderly (aged 74·2 ± s.d. 9·8 years) and mainly female (73%).

Diffusion experiments

In-vitro diffusion studies were performed on an automated diffusion system using miniature diffusion cells with flow-through receptor compartments (Akhter et al 1984). The cells had a diffusional area of 0·125 cm^2 and were equilibrated at 32°C. Sink conditions were maintained by pumping through a degassed aqueous receptor solution at 2 mL h^{-1}. The receptor solution contained 0·002% sodium azide to prevent bacterial growth.

Epidermal membranes were floated, stratum corneum side up, on receptor solution for 48 h before mounting in the diffusion cells, to ensure essentially full hydration.

The drug donor solution was a saturated aqueous solution, at 32°C, of 5-FU, radiolabelled to an activity of approximately 0·1 mCi mL^{-1}. The solubility of 5-FU in water, at 32°C, was determined spectrophotometrically to be 14·3 ± s.d. 0·6 mg mL^{-1} (mean of four replicate measurements). 5-FU is a weak acid (pK_a 8·0 and 13·0) and was therefore largely un-ionized in double distilled water (pH 4·6) (Rudy & Senkowski 1973). The log $K_{octanol/water}$ for 5-FU was determined, using radiolabelled drug, to be −0·92 (mean of three replicate measurements).

For the initial control runs, 200 μL donor drug solutions were dispensed into the cell donor compartments, which were then covered. Receptor solution was collected over 2-h periods for a minimum of 36 h, and mixed with 5 mL Optiphase "Hisafe" 3 scintillation mixture (LKB) before analysis on a Packard Tricarb-460 liquid scintillation counter. Following the control runs, the membrane surfaces and donor compartments were rinsed clean of drug and the donor compartments were then filled with distilled water. The 5-FU remaining in the membranes was left to wash-out into the donor and receptor compartments over a period of 12 h, whilst replacing the distilled water at regular intervals. Preliminary studies have shown that following 12-h washing, the geometric mean 5-FU flux reduces to 15% ± s.e. 4·1/3·2 (n = 20) of the initial pseudo-steady-state flux. Residual drug fluxes following the wash-out period were unlikely, therefore, to affect post-treatment diffusion runs.

The epidermal membranes were then treated with 150–200 μL enhancer or enhancer formulation for 12 h. Many of the sesquiterpene compounds selected for this study were solid at 32°C (melting points are listed in Table 1). Therefore, it was necessary to deliver them saturated in a suitable vehicle. Dimethyl isosorbide was chosen since it had the required solvent characteristics and because it has a relatively small

effect on the rate of 5-FU absorption through human skin (Williams & Barry 1989). Unlike propylene glycol, dimethyl isosorbide does not exhibit synergy in-vivo with lipophilic accelerants such as oleic acid and azone (Bennett et al 1985).

After the treatment period the enhancer was removed by gently blotting with tissue paper. In some instances crystals of solid enhancers had to be removed by a single quick rinse with acetone (Bond & Barry 1988). Drug donor solution (200 μL) was then reapplied for the treated run, and samples were collected as before over a minimum period of 36 h.

In both control and treated runs the depletion of drug from the donor solution was determined to be negligible; zero-order diffusion kinetics could therefore be applied. The pseudo-steady-state rate of drug absorption is related to the epidermal permeability coefficient (K_p) by:

$$K_p = (dm/dt)/C \quad (1)$$

where dm/dt is the drug flux per unit area at steady-state and C is the donor drug concentration. K_p values were calculated from the steady-state rates of 5-FU absorption determined from the slopes of the linear portions of the cumulative amount absorbed vs time profiles.

Testing the reversibility of enhancer effects

Initial permeation experiments revealed that sesquiterpene enhancer effects did not diminish markedly over the 36 h post-treatment period. It was postulated that the poor reversibility may have been related to the possible slow clearance of the enhancers from the stratum corneum. Sesquiterpene compounds are only very sparingly soluble in water and are, therefore, expected to partition poorly into aqueous donor and receptor solutions. In an attempt to improve enhancer clearance, aqueous donor and receptor phases were replaced by ethanol:water (1:1). Ethanol:water (1:1) solutions are commonly used in in-vitro studies to improve the clearance of very lipophilic compounds from the skin.

In these experiments the reversibility of enhancer effects were investigated for over 4·5 days post-treatment. To allow for such long post-treatment diffusion runs, the experimental procedure was changed. Freshly mounted epidermal membranes were treated with enhancers for 12 h, without performing the usual, initial control diffusion runs. 5-FU absorption was then followed for 4·5 days, replacing the saturated drug donor solution after 3 days to prevent depletion. Control K_p values were recorded simultaneously using untreated membranes obtained from the same skin samples.

The solubility of 5-FU in ethanol:water (1:1), at 32°C, was determined spectrophotometrically to be 18·3 mg $mL^{-1} \pm$ s.d. 0·9 (mean of four replicate measurements).

Stratum corneum/water partitioning experiments

Parallel stratum corneum/water partitioning experiments were performed, since it was difficult to measure partitioning changes in the small membranes used in the diffusion experiments. Partitioning changes can be calculated using diffusional lag-times (Williams & Barry 1991b). However, lag-times are highly variable and unreliable diffusion parameters. Furthermore, in this study, lag-times usually increased following treatment with unformulated enhancers, thus making any lag-time calculation highly suspect.

Discs (16 mm diam.) were cut from dried sheets of stratum corneum with a cork borer. Each disc was then floated on a 0·002% aqueous solution of sodium azide for a minimum of 48 h, at 20°C. Fully hydrated discs were floated-out flat onto tissue paper, blotted dry and then immersed in the enhancers. The stratum corneum samples were treated for 12 h, at 20°C, to mimic the treatment period in the permeation studies.

Treated discs were blotted free of enhancer and floated on a 10 mg mL^{-1} aqueous solution of 5-FU (radiolabel activity of 0·01 mCi mL^{-1}) for 24 h at 32°C. Preliminary experiments have shown that both control and sample discs require 24 h to equilibrate fully at 32°C; washing-out of the enhancers over this period was not a problem.

Following equilibration each drug solution was sampled in triplicate. Equilibrated discs were then floated-out flat onto tissue paper, blotted free of drug solution and immediately weighed. Weighed discs were solubilized overnight in 1 mL Soluene-350 (Packard). The Soluene was neutralized with 10 μL glacial acetic acid and mixed with 5 mL scintillant before liquid scintillation counting.

The stratum corneum/water partition coefficient ($K_{sc/water}$) was calculated as the ratio of the radioactive counts (g hydrated stratum corneum)$^{-1}$ to the counts (mL drug solution)$^{-1}$. This hydrated $K_{sc/water}$ value was used since it reflected more closely the partitioning which would have occurred in the permeation experiments. It should be noted that in these partitioning studies, after equilibration, the 5-FU would be evenly distributed with the stratum corneum. In the diffusion studies a concentration gradient would exist across the stratum corneum and the total amount in the membrane would be half that noted in the partitioning studies.

Calculation of results

The overall potency of each enhancer was expressed as a ratio of the K_p value before and after enhancer treatment, thereby establishing each piece of skin as its own control (Goodman & Barry 1988).

$$\text{Enhancement ratio (ER)} = \frac{K_p \text{ after treatment } (K_{pe})}{K_p \text{ before treatment } (K_p)} \quad (2)$$

In studies investigating the reversibility of enhancer actions, control K_p values and post-treatment K_p values were measured using different epidermal membrane samples. In this instance, ER values were calculated from the geometric mean control K_p and the respective geometric mean post-treatment K_p.

The effect of the enhancers on 5-FU partitioning into the stratum corneum was described as a partitioning ratio.

$$\text{Partitioning ratio (PR)} = \frac{K_{sc/water} \text{ after treatment } (K_e)}{K_{sc/water} \text{ before treatment } (K)} \quad (3)$$

Each PR value was measured using control and treated discs from the same cadavers. Since

$$K_p = (K_{sc/water}\, D)/h \quad (4)$$

where D is the apparent drug diffusitivity in the stratum corneum and h is the thickness, it was possible to calculate D

Table 2. The effects of sesquiterpene enhancers on 5-fluorouracil absorption across human epidermis in-vitro. Data are summarized using geometric means and geometric standard errors. Solid enhancers were applied saturated in dimethyl isosorbide.

Enhancer pre-treatment	n	Mean K_p (cm $h^{-1} \times 10^5$)	Mean K_{pe} (cm $h^{-2} \times 10^5$)	Mean ER	Log $K_{octanol/water}$
Hydrocarbons					
(+)-Longifolene	6	1·08 (+0·38/−0·28)	1·79 (+0·29/−0·25)	1·66 (+0·54/−0·41)*	7·10
β-Caryophyllene	4	6·20 (+1·82/−1·41)	12·3 (+4·89/−3·50)	1·99 (+0·24/−0·22)***	6·00
(+)-Aromadendrene	6	2·17 (+1·21/−0·78)	5·53 (+2·87/−1·90)	2·55 (+1·14/−0·79)**	6·55
(+)-Cedrene	5	0·90 (+0·50/−0·32)	2·4 (+0·34/−0·30)	2·67 (+1·04/−0·75)**	6·09
Alcohols					
(−)-Isolongifolol/dimethyl isosorbide	5	4·41 (+1·20/−0·94)	4·16 (+0·55/−0·48)	0·94 (+0·29/−0·22)*	5·14
(−)-Guaiol/dimethyl isosorbide	5	3·82 (+0·78/−0·64)	14·6 (+7·41/−4·89)	3·82 (+2·40/−1·48)**	5·02
(+)-Cedrol/dimethyl isosorbide	5	4·28 (+1·43/−1·07)	19·7 (+11·4/−7·20)	4·60 (+2·66/−1·68)**	5·01
(−)-α-Bisabolol	5	3·88 (+1·02/−0·80)	32·8 (+5·09/−4·41)	8·45 (+1·33/−1·15)***	4·35
Farnesol	6	2·86 (+1·43/−0·91)	40·5 (+30·5/−17·4)	14·2 (+3·85/−3·02)***	3·75
Nerolidol	18	1·00 (+0·24/−0·19)	22·7 (+6·48/−5·04)	22·8 (+4·67/−3·88)***	3·53
Others					
β-Caryophyllene oxide/dimethyl isosorbide	5	6·90 (+2·68/−1·92)	70·4 (+31·6/−22·0)	10·2 (+2·20/−1·84)***	4·73
(+)-Cedryl acetate/dimethyl isosorbide	8	2·11 (+0·51/−0·40)	27·9 (+5·29/−4·51)	13·2 (+4·20/−3·20)***	6·05
Control					
Dimethyl isosorbide	4	3·50 (+0·61/−0·52)	13·0 (+4·17/−3·15)	3·73 (+2·01/−1·31)**	

n, number of replicate measurements. K_p, control permeability coefficient. K_{pe}, permeability coefficient following enhancer treatment. ER, enhancement ratio. Log $K_{octanol/water}$, theoretical octanol/water partition coefficient. *$P < 0·1$, **$P < 0·025$, ***$P < 0·005$.

before and after enhancer treatment. For this calculation the membrane thickness was assumed to be constant (35 μm), i.e. the D value calculated is an apparent value, uncorrected for the tortuosity of the intercellular diffusional pathway. It was also assumed that the stratum corneum provided the major diffusional resistance in the permeation studies, and therefore that $K_{p(epidermis)}$ approximated to $K_{p(stratum\ corneum)}$.

The effect of the enhancers on the apparent drug diffusivity in the stratum corneum was described as a diffusivity ratio.

$$\text{Diffusivity ratio (DR)} = \frac{\text{D after treatment } (D_e)}{\text{D before treatment } (D)} \quad (5)$$

Statistical considerations

Analysis of control permeation data from this and other previous studies revealed that K_p values for 5-FU across human epidermis in-vitro are not Gaussian-normally distributed as is usually assumed (Williams et al 1992). Logarithmic transformation (base 10) of the data produced frequency distributions which were Gaussian-normal. In the present study all permeation and partitioning data were treated as being log-normally distributed. Experimental data were described using geometric means and geometric standard errors (s.e.). Geometric standard errors were calculated by adding or subtracting the logarithmic s.e. from the logarithmic mean, and then anti-logging both values. Since the anti-logged values represented the geometric mean plus or minus the geometric s.e., the actual s.e. values themselves were calculated by adding or subtracting the geometric mean. As a consequence of the log-normality, the geometric s.e. values were slightly asymmetric, with the larger positive errors reflecting the skew of the data distributions. In this study ER and DR values for each enhancer were tested to see if they were significantly greater than one (i.e. no effect) using a paired one-sided Student's *t*-test on the log ER data. Similarly, the significance of partitioning increases was tested using an unpaired one-sided Student's *t*-test on the log $K_{sc/water}$ data.

Results

Permeation studies

Permeation study results are summarized in Table 2. Treatment with hydrocarbon sesquiterpene compounds produced roughly twofold increases in 5-FU absorption. Cumulative permeation profiles in Fig. 3a show that while treatment with hydrocarbon enhancers increased pseudo-steady-state 5-FU flux, diffusional lag-times were not reduced; in fact, in many instances the lag-times increased. It is likely that increased lag-times are due to gradual increases in membrane permeability produced by the slow redistribution of the enhancers within the stratum corneum and consequently a conditioning of the membrane in the early stages of the diffusion process. Similar delayed onsets of action have been observed with other lipophilic enhancers such as long-chain pyrrolidones

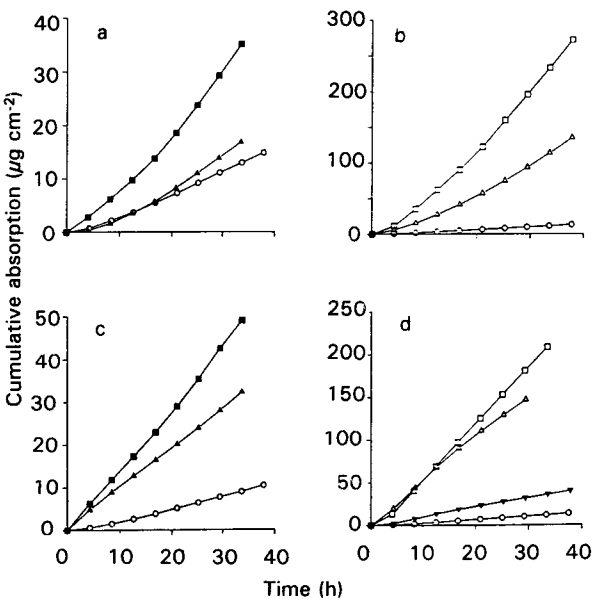

FIG. 3. Typical in-vitro cumulative penetration profiles for 5-fluorouracil permeating human epidermal membranes treated with sesquiterpene enhancers. a. Effect of hydrocarbons, (+)-aromadendrene (■), β-caryophyllene (▲) and control (○). b. Effect of alcohols, farnesol (△), nerolidol (□) and control (○). c. Effect of alcohols saturated in dimethyl isosorbide, (−)-guaiol (▲), (+)-cedrol (■) and control (○). d. Effect of β-caryophyllene oxide saturated in dimethyl isosorbide (△), (+)-cedryl acetate saturated in dimethyl isosorbide (□), dimethyl isosorbide (▼) and control (○). For clarity alternate data points have been omitted.

Table 3. Duration of sesquiterpene enhancer effects on 5-fluorouracil absorption across human skin. Ethanol:water (1:1) donor and receptor phases were used. Data are summarized using geometric means and standard errors.

Enhancer pretreatment	n	Mean initial K_p (cm $h^{-1} \times 10^5$)	Mean K_p after 4·5 days (cm $h^{-1} \times 10^5$)	Mean initial ER	Mean ER after 4·5 days
Control	4	34·9 (+11·4/−8·56)	—	—	—
β-Caryophyllene	6	80·2 (+22·4/−17·5)	80·2 (+22·4/−17·5)	2·29 (+0·64/−0·49)	2·29 (+0·64/−0·49)
(−)-α-Bisabolol	6	123 (+30·6/−24·4)	102 (+22·8/−18·0)	3·53 (+0·87/−0·70)	2·94 (+0·64/−0·53)
Nerolidol	6	441 (+95·5/−77·9)	397 (+72·4/−60·9)	12·6 (+2·73/−2·79)	11·4 (+2·05/−1·77)

K_p, permeability coefficient. ER, enhancement ratio.

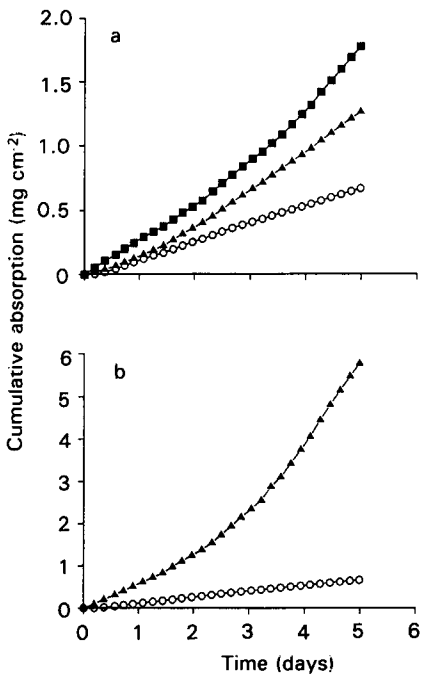

FIG. 4. Typical extended in-vitro cumulative penetration profiles for 5-fluorouracil permeating human epidermal membranes. a. Effects of β-caryophyllene (▲), (−)-α-bisabolol (■) and control (○). b. Effect of nerolidol (▲) and control (○). Experiments used ethanol: water (1:1) donor and receptor phases. For clarity alternate data points have been omitted.

(Sasaki et al 1991) and long-chain fatty acids (Komata et al 1992).

The solid sesquiterpene alcohol compounds delivered saturated in dimethyl isosorbide had weak enhancement effects similar to that of the solvent control. Fig. 3 shows that the sesquiterpene alcohol enhancers saturated in dimethyl isosorbide reduced diffusional lag-times. This effect is probably due to the solvent, since the lag-time in the control was also reduced (Fig. 3d). Treatment with liquid sesquiterpene alcohols produced the best improvements in 5-FU absorption. The geometric ER noted for (−)-α-bisabolol (8·45) agrees well with the arithmetic mean ER of 5·4 reported in previous studies using 5-FU (Kadir & Barry 1991). Nerolidol was determined to be the best enhancer with a geometric mean ER of 22·8. Fig. 3b shows that treatment with unformulated sesquiterpene alcohol enhancers increased diffusional lag-times for 5-FU. As for the hydrocarbon enhancers, this effect is probably related to permeability increases during the post-treatment runs produced by the slow redistribution of the enhancers within the stratum corneum.

The two remaining compounds, β-caryophyllene oxide and (+)-cedryl acetate, both significantly improved 5-FU absorption. It appears that the epoxide and acetate functional groups were more effective than the alcohol group in improving the activities of cyclic sesquiterpenes.

Examination of the post-treatment permeation profiles (Fig. 3) reveals that in each case no marked reversal of enhancement effect occurred over approximately 36 h. It is likely, therefore, that the wash-out of sesquiterpene enhancers from the stratum corneum was very slow when aqueous donor and receptor solutions were used. To investigate the reversibility of sesquiterpene enhancer actions more thoroughly, additional permeation studies were performed using ethanol:water (1:1) donor and receptor solutions. Ethanol:water (1:1) is commonly used to improve the clearance of very lipophilic compounds from the skin.

Testing the reversibility of enhancer effects

Three enhancers were selected for this study; β-caryophyllene, (−)-α-bisabolol and nerolidol. Table 3 summarizes the results obtained using ethanol:water (1:1) donor and receptor phases. Initial K_p values were measured at the onset of pseudo-steady-state diffusion and final K_p values at the end of the experiment after 4·5 days.

The geometric mean control K_p for 5-FU determined using ethanolic donor and receptor solutions ($34·9 \times 10^{-5}$ cm h^{-1}) was approximately 13-fold that of the geometric mean K_p obtained using aqueous solutions ($2·71 \times 10^{-5}$ cm h^{-1}); this represents a flux ratio of 16·5. This study shows, therefore, that ethanol, in combination with 50% v/v water, is an effective penetration enhancer in its own right. These data are in good agreement with studies reported by Berner et al (1989) which have shown that there is an optimal range of 0·5–0·7 volume fraction of ethanol at which nitroglycerin flux across human skin increases up to 10-fold. Increased nitroglycerin flux was shown to be linearly related to increased ethanol flux suggesting that ethanol may increase drug partitioning into the skin. The drop in drug flux over 0·7 volume fraction was believed to be related to a decrease in ethanol flux which in turn was due to stratum corneum dehydration. In support of this model, stratum corneum partitioning experiments have shown increased nitroglycerin uptake into human stratum corneum with increasing ethanol concentration (between 0 and 50% v/v ethanol in water; Berner et al (1989)). A similar maximum in the flux of oestradiol through human skin at 40–60% ethanol has recently been shown by Megrab et al (1993).

The rank order of enhancer activities determined with ethanol:water (1:1) donor and receptor solutions was the same as that reported using an aqueous system (Table 2). These data suggest that the effects of ethanol and sesquiter-

Table 4. The effects of selected sesquiterpene enhancers on 5-fluorouracil partitioning into human stratum corneum and the apparent drug diffusivity in the stratum corneum during pseudo-steady-state absorption across human epidermal membranes in-vitro. Values represent geometric means.

Enhancer pretreatment	Mean K	Mean K_e	Mean PR	*P*	Mean D $\times 10^7$ ($cm^2\ h^{-1}$)	Mean D_e $\times 10^7$ ($cm^2\ h^{-1}$)	Mean DR	*P*
(+)-Cedrene	1·51	1·26	0·84	>0·100	0·20	0·65	3·18	<0·025
β-Caryophyllene	1·06	1·09	1·03	>0·100	1·90	3·84	2·02	<0·005
(−)-α-Bisabolol	1·06	1·66	1·57	<0·025	1·19	6·72	5·63	<0·005
Farnesol	0·53	1·06	2·00	<0·005	1·73	13·3	7·67	<0·005
Nerolidol	0·53	1·17	2·21	<0·005	0·63	6·26	10·0	<0·005

K, $K_{sc/water}$ in control samples. K_e, $K_{sc/water}$ following enhancer treatment. PR, partition ratio. D, apparent diffusivity of 5-fluorouracil in the stratum corneum. D_e, apparent diffusivity following enhancer treatment. DR, diffusivity ratio.

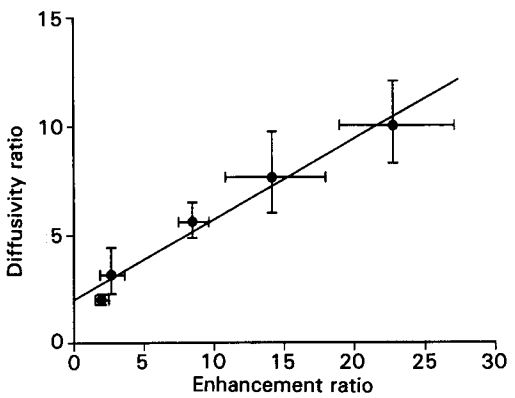

FIG. 5. Relationship between the enhancement ratio, ER, and the diffusivity ratio, DR, for 5-fluorouracil. Values represent geometric means and geometric standard errors.

pene enhancers are simply additive and that no synergy exists between the two. Fig. 4 shows that 5-FU absorption across enhancer-treated epidermis rises gradually over 4·5 days. As discussed above, gradual increases in permeability are probably due to the slow redistribution of the enhancers within the stratum corneum. After 4·5 days all three geometric mean post-treatment K_p values remained significantly higher than control values ($P < 0·005$). No decrease in enhancement effect was observed in β-caryophyllene-treated samples, the mean initial and final ER values being identical. Small decreases in enhancer effects were noted in (−)-α-bisabolol- and nerolidol-treated skin. The reductions did not, however, return K_p values to baseline levels. Sesquiterpene enhancer effects are thus essentially irreversible over 4·5 days, even when enhancer solubilities in the receptor solution are improved with 50% ethanol.

Stratum corneum/water partitioning experiments

Partitioning studies investigated the effects of five sesquiterpenes, β-caryophyllene, (+)-cedrene, (−)-α-bisabolol, farnesol and nerolidol, on 5-FU partitioning into the stratum corneum. Table 4 shows that all three sesquiterpene alcohol enhancers significantly improved 5-FU partitioning. The increase on 5-FU partitioning observed after pretreatment with (−)-α-bisabolol (PR = 1·57) agrees well with the PR previously determined by Kadir & Barry (1991) of 1·11. The hydrocarbon enhancers investigated had no significant effect on 5-FU partitioning. It was noted that, for the five selected enhancers, partitioning increases were linearly related to the overall enhancement effect (PR = 0·0625ER + 0·88, r = 0·95).

Calculation of improvements in drug diffusivity at pseudo-steady-state suggested that an important mechanism of action of each enhancer was to improve drug diffusivity in the stratum corneum (Table 4). The diffusivity and the overall enhancement effects also appeared to be linearly related (Fig. 5) (DR = 1·99ER + 0·37, r = 0·99). A similar relationship, obtained from diffusional lag-times, was observed for a group of monoterpene enhancers (Williams & Barry 1991b).

Discussion

Sesquiterpenes as useful penetration enhancers

Sesquiterpene compounds, which are of low toxicity and cutaneous irritancy (Table 1), have been shown to be effective penetration enhancers for 5-FU in human skin. The best enhancer, nerolidol, increased skin permeability towards 5-FU over 20-fold. Unfortunately, the effects of sesquiterpene enhancers were poorly reversible. Using ethanol:water (1:1) donor and receptor phases it was shown that enhancer effects were almost fully maintained for at least 4·5 days. It is likely that the poor reversibility of sesquiterpene actions is due to the slow release of enhancers from the stratum corneum. Many other lipophilic enhancers may also remain in the skin for extended periods of time. Wotton et al (1985) report, for example, that the effects of Azone (log $K_{octanol/water} = 6·6$) on human skin permeability remain after several days. Similarly, Goodman & Barry (1989) have noted that lipid disruption produced by Azone in human stratum corneum, as detected by differential scanning calorimetry, remains in tissue that has been washed with water for 6 h. It is possible that the main limiting factor in the duration of effect of highly lipophilic enhancers in-vivo is the natural turnover time of the stratum corneum of approximately 14 days.

Mechanisms of action

The mechanisms of action of true skin penetration enhancers have been categorized by Barry (1990) and described using the lipid-protein partitioning theory. The theory suggests that accelerants usually act to alter the lipid or protein structures in the stratum corneum, or to promote drug partitioning into the tissue. This study suggests that sesquiterpenes interact with barrier structures in the stratum

corneum, thus improving 5-FU diffusivity, and that some of the compounds also increase 5-FU partitioning.

The molecular mechanism for the interaction between the sesquiterpenes and the stratum corneum has not been addressed in the present study. It is, however, very likely that these compounds increase 5-FU diffusivity in the stratum corneum by disrupting the intercellular lipid bilayers. Differential scanning calorimetry experiments on human stratum corneum have shown that acyclic terpene alcohols, including farnesol and nerolidol, disrupt lipid bilayers, thus probably increasing their permeability (Cornwell & Barry 1992).

The mechanism behind the increases in 5-FU partitioning following pretreatment with the sesquiterpene alcohols has not as yet been investigated. Why should such very lipophilic compounds improve the partitioning of such a hydrophilic drug? The solubility of 5-FU in nerolidol has been determined, using radiolabelled crystals, to be 0·46 ± s.d. 0·02 mg mL^{-1} (mean of three replicate measurements) at 32°C, i.e. 3% of the aqueous solubility at 32°C. Clearly, partitioning increases cannot be accounted for by simple solvent effects related to the entire stratum corneum; complex formation between terpenes and 5-FU in an alkane domain may be the reason for increased partitioning.

Structure-activity relationships

In general those enhancers with polar functional groups produced the best improvements in the absorption of the model hydrophilic permeant 5-FU. This is in agreement with the work of Williams & Barry (1991b) on the monoterpenes, which also showed that polar functional groups improved accelerant activities towards 5-FU.

It would be expected that the hydrocarbon terpenes would preferentially dissolve in the central portion of the lamellar lipid phase of the stratum corneum, remote from the polar head groups. They would thus have a modest effect in decreasing the viscosity of this domain, which is already somewhat fluid, and thereby enhancing the diffusion of the 5-FU.

Terpenes possessing a polar group have the opportunity to insert into the lipid lamellae with their polar head group aligned with like groups in the stratum corneum, and their alkyl tails similarly apposed to those of the lipids. The space-filling models (Fig. 2) have been orientated to emphasize such alignments. The six alcohol enhancers are almost ideal structural isomers and thus they permit a simple structure-activity analysis to be made. We see that the highly bunched cyclic compounds, (−)-isolongifolol, (−)-guaiol and (+)-cedrol had the weakest enhancing activities of the alcohols (Table 2) and this may be related to their poorer abilities to disrupt the lipids because of the absence of definite hydrocarbon tails. (−)-α-Bisabolol, a monocyclic alcohol, was of intermediate activity and would align better within the lipid domain. The best enhancers were the acyclic alcohols, nerolidol and farnesol. Both these compounds have structures suitable for disrupting lipid packing. Comparison of the structures of nerolidol and farnesol with their activities reveals that changing from a primary to a tertiary alcohol markedly improves enhancer activity. The improvement in activity could possibly be related to the achievement of an ideal ten-carbon chain length (Aungst et al 1986; Hori et al 1991).

β-Caryophyllene oxide, and (+)-cedryl acetate, after allowing for the effect of the dimethyl isosorbide, are poorer enhancers than farnesol and nerolidol; they have a slightly more compacted structure.

Further structure-activity analysis was permitted by calculation of the log $K_{octanol/water}$ ($K_{o/w}$) partition coefficients of the test enhancers using the fragment method of Hansch & Leo (1979); results are in Table 2. It should be noted, however, that the calculated log $K_{o/w}$ values are rough guides; the unusual clustered ring systems in many of the compounds will have unpredictable effects on the true values. In addition, uptake into a structured bilayer is very different from partitioning into water-saturated octanol.

The relationship between the calculated log $K_{o/w}$ values and the goemetric mean ER values is shown in Fig. 6. The arrowed compound is cedryl acetate, which contains an extra methyl group, and is therefore C16 not C15. Fig. 6 suggests that the best enhancers have the lowest log $K_{o/w}$ values. Since chain flexibility, unsaturation and polar groups all decrease log $K_{o/w}$, this trend could just be reflecting a series of structural effects on activities. Conversely, it is also possible that an optimal log $K_{o/w}$ exists for enhancer delivery, as there does for the transdermal delivery of a series of related compounds; however, the series of enhancers used in this work did not include members with low log $K_{o/w}$ values so such a maximum was not evident.

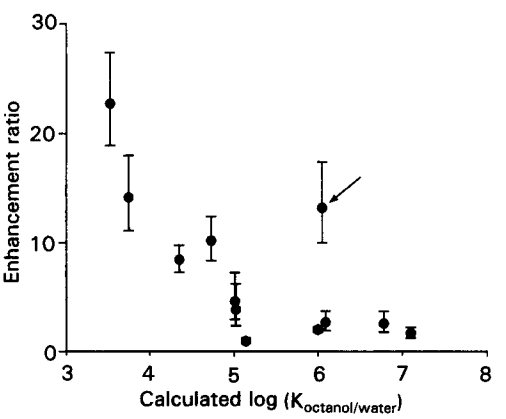

FIG. 6. Effect of theoretical log $K_{octanol/water}$ on the activities of sesquiterpene enhancers (arrowed compound is cedryl acetate; see text).

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