The study was performed to investigate the effect of penetration enhancers on the stratum corneum barrier. Epidermal membranes were prepared from freeze-stored (−70°C) Caucasian breast skin and mounted in a flow-through diffusion cell. The validity of the freeze storage procedure was verified by measurement of [1H]-water penetration. The effect of the cyclic terpene, carveol, on the transdermal penetration of water and ethanol was studied in vitro. Control ethanol and water penetration measured with a donor solution of 50% ethanol/PBS (w/w) was 1.9 ± 0.2 and 3.6 ± 0.5 × 10^−3 cm/h. The addition of 3% carveol to the donor solution increased the permeation of water and ethanol after 4 h to 8.3 ± 1.1 and 12.5 ± 1.9 × 10^−3 cm/h, respectively. In a separate experiment, terpinen-4-ol and α-terpineol were also tested, in addition to carveol, for effect on tritium flux. No significant difference in maximum tritium flux was obtained between the three terpenes studied. The maximum increase in permeability coefficients of carveol, terpinen-4-ol and α-terpineol was 8.7 and 10.9, respectively. Key words: percutaneous absorption; skin penetration enhancers; tritiated water.

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The principal resistance to penetration of substances through intact human skin resides within the stratum corneum, which is comprised of keratin-rich cells embedded in multiple lipid bilayers. We have focused on methods of increasing the stratum corneum permeability by using chemical penetration enhancers. These agents partition into, and interact with, the stratum corneum constituents to ideally induce a reversible increase in skin permeability. Terpene compounds, isolated from plant volatile oils, have been extensively studied as penetration enhancers for human skin (1,2). The chemical structures of the used enhancers are illustrated in Fig. 1.

![Chemical structures of terpenes](image)

Fig. 1. The structural formulae of terpenes used as penetration enhancers.

Surprisingly little interest has been shown in the effect of chemical penetration enhancers on the water barrier function of the stratum corneum. Such studies may illustrate how different penetration enhancers interact with the normal physiological barrier functions of the skin.

In the present study we examined: (i) if the integrity of the barrier function in human skin was retained after freeze storage (−70°C), (ii) the enhanced penetration of [1H]-H2O and [14C]-ethanol in the presence of carveol, and (iii) the effects of carveol, α-terpineol and terpinen-4-ol on the total [1H]-flux on a comparative basis, [1H]-flux representing a combined contribution of [1H] from the flux of water and ethanol. To permit the study of the early phase of enhancer-induced changes in the permeability of the stratum corneum, the penetration enhancers were added to the donor compartment concurrently with the penetrants studied, i.e. [1H]-H2O and [14C]-ethanol.

**MATERIAL AND METHODS**

The model permeants were [1H] H2O and [14C]-ethanol, supplied by NEN Research Products (Dreieich, Germany). The specific radioactivities were 925 MBq/ml and 7.4 MBq/ml. The terpenes used as received were carveol (97%) and terpinen-4-ol (96%) and α-terpineol (99%) (Sigma Chemical Company, MO, USA). The receptor fluid used was a phosphate-buffered saline solution (PBS), pH 7.4. Samples collected, or standards, were brought up to a volume of 2.0 ml by adding distilled water, to which 4.0 ml Ready GelTM cocktail scintillation fluid was added. The radio-labelled penetrant was determined by liquid scintillation counting (Beckman LS 5000 CE scintillation counter). When [1H] and [14C] were measured simultaneously, the [1H]-count had to be reduced due to the registration of [14C] decay in the [1H]-channel of the scintillation counter.

**Flow through diffusion cell**

In vitro studies were performed in an automated system using miniature flow through diffusion cells. The design of the diffusion cells is adapted from the system described by Akhter et al. (3). A support grid, made of 0.40 mm stainless steel and cut by laser, was developed to allow for the mounting of the epidermal membrane in the diffusion cells. The grid had been polished to remove rough edges to reduce the risk of damage to the epidermal membrane. Sink conditions were maintained by a flow of degassed PBS solution through the receptor compartment at 4.0 ml/h. Thermostated blocks, each containing four receptor compartments and maintained at 32.0 ± 0.1°C were placed directly above a Fractomix® autosampler. The cell has a nominal diffusion area of 0.50 cm² (no adjustment was made for the grid). The donor compartments were covered by a teflon plug to prevent evaporation. All plates in contact with the epidermal membrane, donor fluid and perfusion fluid are made of stainless steel, teflon or polyethene. The experiments were performed using two individual blocks with a total of eight diffusion cells per test.

**Preparation of epidermal membranes**

Caucasian breast skin was obtained from plastic surgery. Excess fatty and connective tissues were removed, and the specimens, wrapped in
aluminium foil, were stored at –70°C. Prior to preparation, the skin samples were thawed at room temperature. Thirteen-millimetre diameter disks were obtained using a cylindrical punch. Epidermal membranes were prepared by a heat separation technique (4) by immersing the skin disks in distilled water at 60°C for 90 s, followed by teasing off the epidermal membrane from the underlying dermis with forceps. Specimens rich in appendages (≥ 6 appendages per piece) were discarded to reduce the risk of damage to the membrane during preparation and to avoid excessive transappendageal penetration. The epidermal membranes were collected on the stainless steel support and temporarily stored for hydration on a filter paper soaked with PBS.

When mounted in the diffusion cell, the epidermal membrane had an additional grid placed on top. Sealing between the grids and the diffusion cell bodies was obtained by two Panfiluml® gaskets.

Barrier verification

Prior to all experiments, the integrity of the barrier layer was verified for each epidermal membrane by the determination of water permeability. The water permeability constant (Kw) for skin was used as an index for permeability, as described by Harrison et al. (5). One hundred microliters of a [3H]-H2O solution (9.25 MBq/ml) was applied in the diffusion chambers. Perfusion media, 4.0 ml/h, was sampled at 15-min intervals for 1.5 h. The [3H]-H2O solution was washed from the membrane with PBS solution for a minimum of 3 h, whilst replacing the donor solution at regular intervals. This step reduces the residual [3H]-activity to background levels. Membranes with a water permeability of < 5.0 μl/min/cm² were accepted for the enhancer experiments.

To verify if the use of freeze storage of skin samples was feasible, a separate study was performed. The [3H]-water permeability of a sample of skin was measured in epidermal membranes prepared from fresh skin (< 3 h after surgery). Additional epidermal membranes were collected on grids and stored at –70°C for 2 weeks before measuring water permeability. The remaining skin samples were stored at –70°C. After 2 and 4 weeks of storage, respectively, the skin samples were thawed; epidermal membranes were prepared and the water permeability was measured.

Diffusion experiments:

The effect of carboxyl on the penetration of ethanol and water was measured by applying 100 μl of a homogenous enhancer solution (w/w) of 3% carboxyl, 47% [14C]-ethanol (49 KBq/ml) and 50% [3H]-H2O/PBS (41 KBq/ml) in the four donor chambers. One hundred microliters of a control solution (w/w) of 50% [14C]-ethanol and 50% [3H]-H2O/PBS was applied in the remaining four donor chambers. The 3% level of enhancer was chosen on the basis that it is the maximum concentration of terpenes allowing for homogenous solutions with the same levels of PBS and ethanol for all three tested terpenes. All epidermal membranes for the penetration of ethanol and water were prepared from the same skin source. Samples were collected at 15-min intervals for 4 h. At the end of the period, 10 μl of the applied solutions was collected for analysis. All the samples were determined as above by liquid scintillation counting.

The use of [3H]-H2O as a tool for water flux determination represents an approximation. It is assumed that in a situation with no other proton-donor than water and a near neutral pH, the measured [3H]-flux reflects the water flux. Due to the rapid exchange of protons, the penetration of [3H] no longer only reflects the water flux in the presence of an additional penetrating proton-donor, e.g. ethanol. Where no separate measurement of the ethanol flux is made by the use of [14C]-ethanol, the results are given as the total tritium flux, representing a combined contribution of [3H] from the flux of water and ethanol. To calculate the flux of [3H]-H2O, the exchange of [3H] between the two hydrogens of water and the hydroxyl groups of ethanol and carboxyl, respectively, must be taken into account. The distribution of [3H] between these sites is assumed to be instantaneous and strictly reflecting the molar ratio of ionizable hydrogen. For the test solutions, water accounts for 84% of the ionizable hydrogen and ethanol for 16%. The true value for [3H]-H2O was obtained by subtracting the contribution of [3H] originating from [3H]-[14C]-ethanol based on the amount of ethanol determined by [14C]. The contribution of [3H] from penetrating [3H]-carboxyl (0.3%) is estimated to be negligible and is not compensated for.

Additional experiments were performed to assess the overall effect on the epidermal barriers of two more terpenes, terpin-4-ol and α-terpineol, by measuring the total [3H]-flux. As this test was performed on a different skin sample, carboxyl was also included. All epidermal membranes in this test were prepared from the same source of skin. For each terpene tested, 100 μl of a homogenous enhancer solution (w/w) of 3% terpene, 47% ethanol and 50% [3H]-H2O/PBS (8.2 MBq/ml) was applied in four chambers. One hundred microliters of a control solution (w/w) of 50% ethanol and 50% [3H]-H2O/PBS was applied in the remaining four chambers.

Statistics

Statistical testing for differences in mean values was made by Student's t-test (unpaired, two-tailed) and regression analysis of time series of flux data by PROC GLM (SAS Institute Inc.), p < 0.05 was considered as significant difference. Values are reported as means ± SEM.

RESULTS

Barrier verification

The overall mean value for water permeability under control conditions obtained with our system was 2.7 ± 0.1 × 10⁻³ cm/h (n = 179). There was no statistically significant difference of water permeability between the epidermal membranes prepared from fresh skin, those kept frozen for 2 weeks after preparation, or those prepared after 2 or 4 weeks of freeze storage (Table 1).

Penetration of ethanol and water

The end value of 1.9 ± 0.2 × 10⁻² cm/h for ethanol with the solution of 50% ethanol/PBS (w/w) is in agreement with data reported by Berner et al. (6). Ethanol alone had a moderate enhancing effect on water flux and the values obtained differ significantly from the values obtained during barrier verification (p < 0.05, Student's t-test). Neither ethanol nor water did, however, reach a true steady-state during the 4-h sampling period but showed a continuing increase (Fig. 2). The end value for water was 3.6 ± 0.5 × 10⁻³ cm/h. Calculated as an enhancement factor for water, compared to the control flux obtained during barrier verification, the value was 1.6. In the presence of 3% carboxyl, both ethanol and water showed a significant increase in flux, compared to the vehicle control (p < 0.05, Student's t-test and GLM). The process was slow, and the flux of both ethanol and water was still increasing at the end of the sampling period. The end values of the flux of

Table 1. The effect of freeze storage (–70°C) on human skin permeability to water

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Mean flux cm/h × 10⁻³, n = 8</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh skin (&lt; 3 h after surgery)</td>
<td>4.30</td>
<td>0.41</td>
</tr>
<tr>
<td>Epidermal membranes frozen 2 w</td>
<td>3.45</td>
<td>0.35</td>
</tr>
<tr>
<td>Skin freeze-stored 2 w</td>
<td>3.84</td>
<td>0.30</td>
</tr>
<tr>
<td>Skin freeze-stored 4 w</td>
<td>3.44</td>
<td>0.29</td>
</tr>
</tbody>
</table>

Kp values are given as cm/h × 10⁻³.

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Penetration of water and ethanol through human epidermal membranes with and without the terpene carvone. Filled symbols represent the values for flux measured in the presence of 3% carvone in combination with 50% ethanol and 47% PBS. Open symbols represent the values for the vehicle control of 50% ethanol/PBS. Values are means ± SEM, n=4 (replicates from the same skin source).

ethanol and water were 8.3±1.1 and 12.5±1.9×10⁻⁹ cm²/h, respectively. Expressed as an enhancement factor, compared to the flux in the presence of ethanol and water (PBS alone), the corresponding values were 4.4 and 3.5. The increase of permeability for ethanol is thus approximately 25% higher than that for water. Expressed as an enhancement factor for water compared to the control flux obtained during barrier verification, the value was 5.4. Calculated as the total tritium flux, the end values are 25.3±3.5×10⁻⁹ cm²/h in the presence of carvone and 6.4±0.7×10⁻⁹ cm²/h for the vehicle control. This is in agreement with the values obtained with a different skin source (see below).

A complicating factor in finite dose experiments is the possible change in the donor fluid composition due to loss of substance by penetration and dilution through the influx of a receptor fluid. Integration of the ethanol and water flux shows that in the presence of carvone, on average 6.6% of the [¹⁴C]-ethanol and an estimated 14.4% of the [³H]-activity was lost from the donor compartment through penetration. For the control vehicle, the corresponding values were 2.6% and 5.9%, respectively. These differing rates of penetration between [¹⁴C]-ethanol and [³H] were not reflected in the activity of the 10 µl samples taken from the donor compartment at the end of the 4 h period. The overall reduction of the activity in the donor compartment was interpreted as an effect of dilution due to a flux through the epidermal membrane of water from the receptor to the donor compartment. The flux values reported in this study were not compensated for this gradual dilution of the solution in the donor compartment. If compensated, the end values would have to be adjusted by a factor of approximately 1.4.

Penetration of total tritium

The control solution of 50% ethanol/PBS showed a slow increase of total tritium flux over time and reached an end value of 5.2±0.9×10⁻⁹ cm²/h (Fig. 3). The tritium flux measured during the sampling period showed similarities between the three terpenes studied. In the presence of carvone, the tritium flux increased during the whole of the period, whereas both terpine-4-ol and α-terpineol displayed a maximum with a decrease during the latter part. The obtained maximum values of [³H]-flux for carvone, terpine-4-ol and α-terpineol were 24.4±1.1 (4.0 h), 20.0±4.2 (2.5 h) and 25.1±2.0 (3.25 h)×10⁻⁹ cm²/h, respectively, and did not differ significantly (p>0.05, Student's t-test). In comparison with pure [³H]-H₂O/PBS solution, the flux calculated as maximum enhancement factor was found to be 10.6, 8.7 and 10.9, respectively. Expressed as maximum enhancement factor for tritium flux compared to flux in the presence of the vehicle control, the corresponding values for carvone, terpine-4-ol and α-terpineol were 4.7, 3.8 and 4.8, respectively. At the end of the exposure period, the activity of [³H] in the donor compartment was significantly lower in compartments containing terpene compared to those of the vehicle controls (p<0.05, Student's t-test). The respective values were on average 60% and 80% of the initial activity.

DISCUSSION

Two basic parameters in evaluating a possible penetration enhancer are the maximum penetration enhancement obtainable and the time scale for the effect. The model chosen for the studies presented here is one where penetration enhancer is introduced concurrently. The set-up is relevant for the situation where a drug is transdermally administered via a patch containing, in addition to the drug, a penetration enhancer.

Any result must be based on a system where the validity of each epidermal membrane is controlled. In the present study this was done by verifying both the utility of the freeze storage of the skin and the measurement of water flux in each preparation prior to the onset of the experiment. The skin used in our study was Caucasian breast skin, and the values for water flux obtained are in agreement with previously reported data for human skin (5.7–8). The assessment of retained barrier properties for each epidermal membrane by measurement of water permeability with the use of [³H]-H₂O was shown to be an effective tool to identify defective epidermal membranes prior to tests. In addition, the water penetration
data thus obtained allow for the normalisation of flux data for each membrane by comparing the penetration of other compounds with the basic value of water flux.

The enhancement by monoterpenes on the penetration of 5-fluorouracil and oestradiol has been reported by Williams & Barry (1,2). These authors found that terpenes with polar groups (alcohols and ketones) are more effective enhancers in the case of the polar 5-fluorouracil than the lipophilic oestradiol. Carveol was reported to induce an approximately 20-fold increase in 5-fluorouracil following a 12-h pre-treatment with pure terpene. The enhancement factor of 5.4 for water obtained in the presence of carveol in this study is considerably lower. This may be the result of the low carveol concentration applied (3%) and the short duration of the experiment, compared to a long pre-treatment period with pure substance. This assumption is supported by data on water penetration following pre-treatment with carveol (unpublished data). In addition, as the aim of the present work was to study the initial phase of penetration changes induced by terpenes, the result is not directly comparable to the steady-state conditions reported by Williams & Barry (1,2).

It was an expected observation that there would be no significant difference of maximum [H]-flux between the three terpenes carveol, terpinen-4-ol and α-terpineol during the period of 0 to 3 h. This is due to the similar physical/chemical properties of the terpenes utilized in this study. The observed maxima of [H]-flux in the presence of terpinen-4-ol and α-terpineol, compared to the continuous increase of carveol, are not interpreted as a reduction of permeability. They are assumed to be mainly caused by the dilution of the donor compartment due to the influx of water from the receptor compartment, thus reducing the [H]-activity. The data indicate a more rapid increase in penetration in the presence of terpinen-4-ol and α-terpineol compared to carveol. This would probably result in an earlier dilution of the [H]-activity of the donor compartment compared to carveol. The dilution of the donor compartment is an effect of the water flux through the epidermal membrane and not of a leakage in the system. This is indicated by the significantly higher dilution in the presence of terpenes compared to the vehicle controls.

The mechanism by which polar compounds move across the stratum corneum is still a subject of debate (9,10). The major penetration route through the stratum corneum appears to be intercellular, around the corneocytes within the lipid domain. A penetration enhancer, such as carveol, which interacts with the lipid bilayers of the stratum corneum, could effect permeability for all substances penetrating by this route. However, our data suggest that there can be quantitative differences in this effect between penetrants with different properties. This is illustrated by the approximately 25% higher increase in penetration for ethanol compared to the smaller and more polar water molecule. Understanding the mechanism for these differences could make possible the use of the effects on water penetration as a predictive tool for the penetration of other polar penetrants.

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