**ABSTRACT**

An in vitro human skin absorption study was conducted on Geranyl nitrile (GN), a widely used fragrance ingredient. Skin permeation and distribution of GN was determined using epidermal membranes from cosmetic surgery donors. Skin membranes were mounted into Franz-type diffusion cells with the stratum corneum facing the donor chamber. The average area available for diffusion was 1.3 cm². The test material (GN) was applied under non-occlusive conditions, at the maximum in-use concentration of 1% in 70/30 (v/v) ethanol/water to the skin surface at a target dose of 5 μl/cm². Permeation was measured over 24 h at 12 time points, using 6% (w/v) Gelfoam 20 PBS as receptor. At 24 h, 1.89 ± 0.16 μg cm² of GN had permeated from the applied dose of 3.74 ± 0.30 μg (mean ± standard error, n=12). Following rapid initial permeation, the rate began to plateau, due to depletion of the donor phase through evaporation. The 24 hour surface water wash/skin mass/weight contained 6.23 ± 0.16% and 3.4 ± 0.22% of the applied dose, respectively. The stratum corneum tape strips and the epidermis (including any in the remaining stratum corneum after tape stripping) contained 1.33 ± 0.16% and 0.41 ± 0.06% of the applied dose, respectively. The potential evaporation loss of GN was estimated by measuring the loss from PTFE (polytetrafluoroethylene) sheets. The evaporation loss of GN from PTFE sheets, under the same experimental conditions was 22% over 24 hrs. Recovery of GN at 24 h was low at 14.1 ± 0.4% due to evaporation. The total absorbed dose value of 4.72 ± 0.32% was computed by combining the levels of GN in the epidermis, stratum corneum tape strips and the receptor fluid. The results indicate that levels of GN permeated due to its rapid evaporation. Based on data in this study, the systemic exposure resulting from the use of GN as a fragrance ingredient would be expected to be low.

**METHOD**

Phosphate buffered saline (PBS) with pH = 7.4 and 4% (w/v) Brij 98 (Gotech 20) in PBS was prepared. The BPRB receptor was determined to be a suitable receptor phase for GN and PBS was the suitable receptor for BA.

The epidermal membranes were prepared from full thickness human female abdominal skin obtained from cosmetic surgery donors using an industry accepted standard procedure. On the day of experiment the epidermal membranes were floated onto water, placed on filter paper (36 mm) and then mounted as a barrier between the halves of reconstituted epidermal microvascular skin (Schematic 1) with 1.3 cm² average area available for diffusion. The receptor chambers were maintained at 37.0 ± 0.5ºC. Prior to dosing the membrane integrity of the cells was evaluated using trypan blue and all cells exhibited normal permeability. The cells were allowed to temperature equilibrate for 30 mins. The diffusion cell was placed on an electronic balance (Sartorius BP211D5) and GN at a concentration of 1% ethanol was applied to the skin surface at a target dose of 5 μl/cm². The actual weight of GN applied was recorded, and converted to the exact volume applied density (0.8700 g/ml). Twelve replicate wells were dosed with 1% geranyl nitrile solution in ethanol (70%) and three control wells were dosed solely with ethanol (70%). Subsequently, benzoic acid (reference) at 0.4% concentration (w/v) ethanol:water (1:1) was applied to the skin surface in the same manner at a target dose of 25 μl/cm². Diffusion cell donor chambers for the reference compound were immediately sealed with parafilm. These selective conditions for the reference were necessary to enable comparison with published data from a multicenter study. The average applied doses (mean ± standard error, n=8) for geranyl nitrile, control groups and the reference compound (8 replicates) were 5.05 ± 0.09, 5.36 ± 0.19 and 5.24 ± 0.74 μl/cm², respectively.

Schematic 1: Representation of the In-vitro Skin Permeation Protocol

**DETERMINATION OF SKIN PERMEATION**

Using a digital pipette, 200 μl samples were taken at 12-timepoints from each receptor chamber of the geranyl nitrile dosed cells, at 0, 1, 2, 3, 4, 6, 8, 10, 12, 16, 20 and 24 hours after dosing. Each sample was placed into scintillation fluid and analysed for 14C using Wallac 1409 LSC liquid scintillation counter. Similarly the receptor phase samples of the benzoic acid (reference) dosed cells were removed at 1, 2, 3, 4 and 5 hours and counted.

**DETERMINATION OF SKIN DISTRIBUTION**

After the removing samples from the receptor chamber of the geranyl nitrile dosed cells, the diffusion cells were dismantled, and the epidermal membranes were scored onto a small disc of thin plastic using cryoanalytical adhesive. The skin surface was wiped with a dry cotton bud to remove any geranyl nitrile residue. The epidermal membranes were tape stripped 10 times using adhesive tape to remove geranyl nitrile. The tape strips were grouped and dissolved in the same vial in Optimix.

**DETERMINATION OF SKIN DISTRIBUTION**

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**MATERIALS AND METHODS**

This study was conducted according to the Scientific Committee on Cosmetic Products and Non-Food Products (SCCNFP) guidelines for in vitro percutaneous absorption assessment (SCCNFP, 2000).²

**MATERIALS**

GN (lot number 007655-6) and 2-14C-GN in ethanol was supplied by BASF (Germany). Liquid scintillation cocktail, Cyanoacrylate and Super Glue (Germany) were obtained from the Research Institute for Fragrance Materials, Inc. \( ^{a} \) (RIFM). 14C and 3H benzonic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tritiated water and (7-14C)-benzoic acid in ethanol were purchased from ICN Biomedicals (Aurora, OH, USA).

**EQUIPMENT**

Sartorius BP211D5 analytical balance and Wallac 1409 scintillation counter were the equipments used for radiolabelling. The Wallac 1409 scintillation counter was used to analyze and measure the isotope distribution. An in-vitro human skin permeation and distribution of geranyl nitrile (GN) was determined using epidermal membranes following GN application (5μl/cm²) in ethanol (70%), under non-occlusive conditions, at a maximum in-use concentration (UN). For percutaneous absorption, stratum corneum is the rate limiting step. The optimal characteristic for this weight was converted to exact volume. The Wallac 1409 scintillation counter was used to analyze and measure the isotope distribution. An in-vitro human skin permeation and distribution of geranyl nitrile (GN) was determined using epidermal membranes following GN application (5μl/cm²) in ethanol (70%), under non-occlusive conditions, at a maximum in-use concentration (UN). For percutaneous absorption, stratum cornueum is the rate limiting step. The optimal characteristic for this weight was converted to exact volume.
remaining skin epidermis (containing any stratum corneum) was also dissolved in OptiSolv. The remaining skin epidermis and the strips, at 200 μl each, were analysed for 14C using LSC (liquid scintillation counter). The diffusion cell donor chambers were also wiped using cotton buds to remove grease. The grease was dissolved with tetrahydrofuran and samples were analysed using LSC. Subsequently, the cotton bud, membrane filter paper supports and donor chambers were washed and/or extracted with acetonitrile and were analysed for 14C using LSC.

ASSESSMENT OF EVAPORATIVE LOSS OF FRAGRANCE MATERIALS

The evaporative loss of GN under experimental conditions was assessed by assembling five ungreased diffusion cells and replacing the skin membranes with PTFE (polytetrafluoroethylene) sheets. GN solution at 1% concentration in 70% ethanol was applied at dose of 5 μl (mean ± SE = 5.01 ± 0.27 μl). The cells were dismantled singly at 1, 2, 6, 12 and 24 hours and the PTFE sheet was removed. The PTFE sheet and donor chamber were washed with acetonitrile. A sample of each wash solution was counted to allow calculation of the remaining radiolabel at each time point.

Calculation of Results

As per SCCNFP guidelines (SCCNFP)3 amount of GN (μg/cm²) and the applied dose of GN (μl) in the receptor phase and various compartments of the skin and the diffusion cell were calculated. A total absorbed dose value was produced by combining the levels of GN in epidermis, plus any remaining in stratum corneum after tape stripping, filter paper membranes support and receptor fluid.

RESULTS

Table 1: Overall recovery (% applied dose) of geranyl nitrile from PTFE surfaces and donor chambers

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Test Material</th>
<th>Average GN dose (μg/cm²)</th>
<th>Recovery (%)</th>
<th>Final Recovered (24-h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Five PTFE</td>
<td>1% GN in 70% ethanol</td>
<td>50.3 ± 2.7</td>
<td>35.7 (1-h)</td>
<td>10.4 (2-h)</td>
</tr>
</tbody>
</table>

Figure 2: Overall recovery (% applied dose) of GN from PTFE surfaces and donor chambers

3.1 Evaporative loss

The evaporative loss of GN was assessed using PTFE cells. It was the major reason for the low recovery of applied GN in the skin absorption assessment. The average GN dose was 50.3 ± 2.7 μg. The recovery (% at 1 and 2 h) was 35.7% and 10.4%. The combined recovery from PTFE membranes and the donor chamber is shown in Figure 2, decreases as the time increases, due to evaporation of the test material. The remaining material was gradually reduced and a total of 7.1% applied dose recovered at 24-hours.

Figure 3: Skin Permeation of GN

3.2 Skin Permeation of GN

In Table 2, the average dose of GN for the twelve active cells was 50.7 ± 0.58 μg/cm² due to rapid loss by evaporation the recoveries of applied GN were low (14.1 ± 0.6%). Permeation of GN as shown in Figure 3, was 1.90 ± 0.15 at 24-h. The 24-h permeation values for the exact dose applied to each cell, after normalisation were 3.74 ± 0.30. The distribution of GN within the skin at 24-h was determined by measuring levels of GN within the stratum corneum tape strips and the epidermis and any remaining in the lower stratum corneum (following tape stripping). The individual tape strip group data together with other distribution and permeation data are shown in Table 3. The overall skin absorption of GN as defined by the SCCNFP was 2.34 ± 0.16 μg/cm² or 4.72 ± 0.32%. The amount in the stratum corneum (tape strips) at 24-h were not considered as absorbed and contributing to the systemic dose.

Table 3: Skin Distribution of GN

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Test Material</th>
<th>Average GN dose (μg/cm²)</th>
<th>Permeated (μg/cm²)</th>
<th>Recovery (%)</th>
<th>Final Recovered (μg/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Twelve active cells</td>
<td>1% GN in ETOH</td>
<td>50.7 ± 0.9</td>
<td>1.90 ± 0.15</td>
<td>3.74 ± 0.30</td>
<td>7.15 ± 0.30</td>
</tr>
</tbody>
</table>

The recovery of GN is presented in Figure 2. The amount of GN recovered is inversely proportional to time. Hence, the recovery is lowest at 24-h and highest at 0-hours. This inversely proportional relationship is most likely due to rapid evaporation of GN, as this test was conducted under non-occluded conditions.

DISCUSSION

Based on the results in this study, the absorption of GN through human skin was low. A total absorbed dose value of 4.72 ± 0.32% was produced by combining the levels of GN (as per SCCNFP guidelines) (SCCNFP, 2000)3 in epidermis, plus any remaining in stratum corneum after tape stripping, filter paper membranes support and receptor fluid. Due to rapid evaporation the overall recoveries were 14.1 ± 0.4%.

The annual dose levels for GN are 1 to 100 metric tons (IFRA, 2000)4. However, in perumary the maximum skin level of GN in a formula that gets into free fragrance has been reported to be 0.34% (IFRA, 2000)4, of which would result in a maximum daily exposure on the skin of 0.02 mg/kg for high end users of these products. Systemic absorption of GN including the epidermis as a sink was observed to be 4.72 ± 0.32% of the applied dose. The absorption of GN through human skin under non-occluded conditions is low. Hence, systemic exposure to GN when used as a fragrance ingredient would be quite low.

The overall recovery at the high end of the absorption rates reported in the reference permeant (BA) with the measurement values in a multicenter study. As presented in Table 4, our experimental BA permeation (32.4 μg/cm² h) fell in the range of the permeation rates reported in the multicenter study (van de Sandt et al., 2000).5

Table 4: Absorption of the reference permeant (BA)

<table>
<thead>
<tr>
<th>Reference permeant</th>
<th>Experimental results</th>
<th>Multi-centre study results</th>
<th>Multi-centre study highest absorption rates</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA (Benzoic Acid)</td>
<td>32.4 μg/cm² h</td>
<td>2.87 to 38.20 μg/cm² h</td>
<td>30 to 32 μg/cm² h</td>
</tr>
</tbody>
</table>

The skin distribution of GN in the GN dosed cells in the epidermis, filter paper and the apparatus was determined and presented. The epidermis was stripped with 1-10 strips. The highest skin distribution of the GN applied dose (%) was found in the wiper.

3.3 Reference permeant data

The validity of the test systems was confirmed by comparing our experimental absorption of the reference permeant (BA) with the measurement values in a multicenter study. As presented in Table 4, our experimental BA permeation (32.4 μg/cm² h) fell in the range of the permeation rates reported in the multicenter study (van de Sandt et al., 2000).6

ACKNOWLEDGMENTS

This study was funded by the Research Institute for Fragrance Materials. The diffusion cell image used in this poster is courtesy of PermeGear Inc. Special thanks to A.M. Api, C.S. Letizia and J. Lalko for their support.

REFERENCES

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Table 5: Skin Permeation and recovery of GN

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