IN-VITRO HUMAN SKIN PENETRATION OF THE FRAGRANCE MATERIAL GERANYL NITRILE

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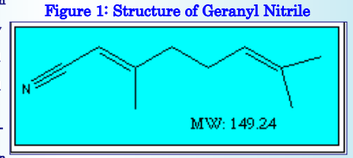
ABSTRACT

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 1.2 cm^2. 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/cm^2. Permeation was measured over 24 h at 12 time points, using 6% (w/v) Oleth-20 PBS as receptor. At 24 h, 1.89 ± 0.15 µg/cm^2 of GN had permeated from the applied dose of (3.74 ± 0.30%) (mean ± standard error, SE, n=12). Following rapid initial permeation, the rate began to plateau, due to depletion of the donor phase through evaporation. The 24 hour surface wipe and donor chamber wash/wipe contained 6.23 ± 0.16% and 1.84 ± 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 0.416 ± 0.050% of the applied dose, respectively. The potential evaporative loss of GN was estimated by measuring the loss from PTFE (polytetrafluoroethylene) sheets. The evaporative loss of GN from PTFE sheets, under the same experimental conditions was 93% over 24 h. Overall 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, filter paper membrane support and receptor fluid. The results indicate that low 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.

INTRODUCTION

Geranyl nitrile (geranonitrile, 3,7-dimethyl-2,6-octadienenitrile, CAS No. 5146-66-7) [GN] is used in perfumery. It has been in public use since the 1940s. GN has not been reported to occur in nature. It can be synthesized from geranialoxime with acetic anhydride (Arctander,1969)[1]. As a fragrance ingredient, it is appreciated for its lemony-fresh odor similar to citral (Calkin and Jellinek, 1994)[2]. GN is used extensively as a fragrance ingredient in wide array of consumer products. It is incorporated in soaps, shampoos, cosmetics, perfumes, detergents, insect repellents and various other consumer products. In an attempt to determine its toxicity it is first important to understand its permeation and metabolism. An *in-vitro* human skin permeation and distribution of geranyl nitrile [GN] was determined using epidermal membranes following GN application (5µl/cm^2) in ethanol (70%), under non-occlusive conditions, at a maximum in-use concentration (1%).

Figure 1: Structure of Geranyl Nitrile



For percutaneous absorption, stratum corneum is the rate limiting step. The optimal characteristic for percutaneous absorption is that the permeant should be reasonably soluble in hydrophilic and hydrophobic media. The rate of diffusion of a non-polar permeant is proportional to its lipid solubility and is inversely related to its molecular weight. GN has a molecular weight of 149.24, low calculated water solubility (~45 mg/l) and a calculated octanol/water partition coefficient, log Ko/w of 3.47. These characteristics suggest that geranyl nitrile would permeate readily through the skin. Eventually, the absolute amount of any compound permeating the skin will also be dependent on the vehicle in which the compound is applied. However, in *in-vitro* studies it is also necessary for the receptor phase to be capable of solubilising the entire permeating compound. To improve the receptor phase solubility of hydrophobic compounds, Oleth-20, a non-ionic surfactant was added to PBS (phosphate buffered saline) at 6% (w/v)

The purpose of this study was to determine the skin permeation and distribution of GN when applied at a maximum in-use concentration of 1% using human tissue *in vitro*. The permeation of benzoic acid [BA], a reference compound was also assessed to confirm the validity of the test systems.

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).[3]

MATERIALS

GN (lot number 00/0316-4) and 2-^{14}C-GN in ethanol was supplied by BASF (Germany).

Solvents used were ethanol (purity 99.7-100%), acetonitrile, tetrahydrofuran of AnalaRgrader or better. Chemicals used for radiolabelling were Tritiated water and (7-^{14}C)-benzoic acid in ethanol.

EQUIPMENT

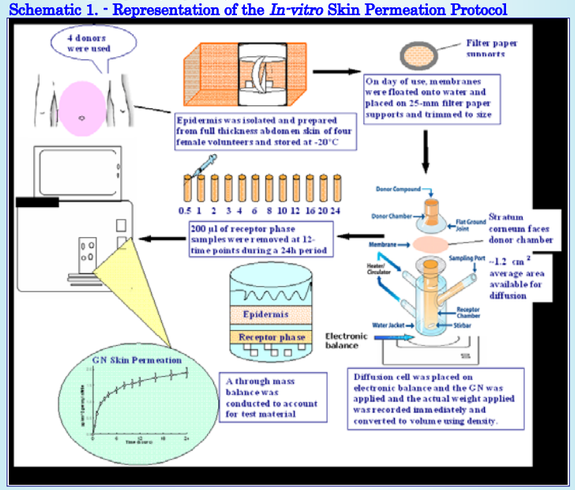
Sartorius BP211D5 analytical balance and Wallac 1409 scintillation counter were the equipments used. The Sartorius analytical balance was used to measure the weight of GN added and using density this weight was converted to exact volume. The Wallac 1409 scintillation counter was used to analyze and measure the ^{14}C in the GN dosed cells. The OptiPhase 'HiSafe' 3 liquid scintillation cocktail, Optisolve tissue solublizer were the reagents used with this equipment.

METHOD

Phosphate buffered saline (PBS) with pH = 7.4 and 6% (w/v) Brij 98 (Oleth 20) in PBS was prepared. The BPBS 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 (25 mm) and then mounted as a barrier between the halves of greased horizontal Franz type-diffusion cells (Schematic 1) with 1.2 cm^2 average area available for diffusion. The receptor chambers were maintained at 37.0 ± 0.5°C so that the skin surface was maintained at 32.0 ± 1°C. Prior to dosing the membrane integrity of the cells was evaluated 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 1% concentration in 70% ethanol was applied to the skin surface at a target dose of 5 µl/cm^2. The actual weight of GN applied was recorded, and converted to the exact volume applied using density (0.8700 g/ml). Twelve replicates were dosed with 1% geranyl nitrile solution in ethanol (70%) and three control cells 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/cm^2. Diffusion cell donor chambers for the reference compound were immediately occluded using greased coverslips. These occlusive conditions for the reference were necessary to enable comparison with published data from a multicenter study. The average applied doses (mean + standard error, SE) for geranyl nitrile, control groups and the reference compound (6 replicates) were 5.05 ± 0.09, 5.36 ± 0.19 and 24.24 ± 0.74 µl/cm^2, 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 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 16, 20 and 24 hours after dosing. Each sample was placed into scintillation fluid and analyzed for ^{14}C using Wallac 1409 LSC (liquid scintillation counter). Similarly the receptor phase samples of the benzoic acid (reference) dosed cells were removed at 1, 2, 4, 8 and 24 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 membrane was secured onto a small disc of thin plastic using cyanoacrylate 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 OptiSolve. The remaining skin epidermis (containing any stratum corneum) was also dissolved in OptiSolve. The remaining skin epidermis and the strips, at 200 µl samples each, were analyzed for ^{14}C 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 analyzed using LSC. Subsequently, the cotton bud, membrane filter paper supports and donor chambers were washed and/or extracted with acetonitrile and were analyzed for ^{14}C 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 membrane with PTFE (polytetrafluorethylene) sheeting. 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/cm^2) and the applied dose of GN (%) 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 membrane support and receptor fluid.

RESULTS

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

Cell type	Test Material	Average GN dose	Recovery (%)	Final Recovered (24-h)
Five PTFE	1% GN in 70% ethanol	50.3 ± 2.7 µg/cm^2	35.7 (1-h) 10.4 (2-h)	7.1%

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

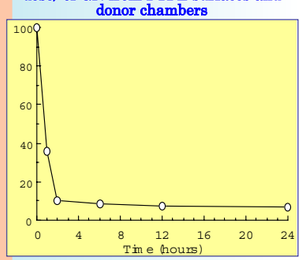


Figure 3: Skin Permeation of GN

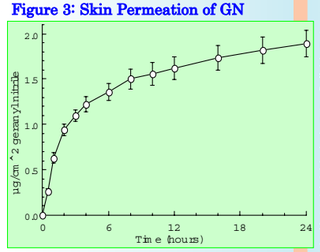


Table 2: Skin Permeation and recovery of GN

Cell type	Test Material	Average GN dose (µg/cm^2)	Permeated (µg/cm^2)	Permeated (%)	Recovery (µg/cm^2)	Final Recovered (%)
Twelve active cells	1% GN (w/v) in EtOH (70%)	50.7 ± 0.9	1.90 ± 0.15	3.74 ± 0.30	7.15 ± 0.3	14.1 ± 0.4%

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.

3.2 Skin Permeation of GN

In Table 2, the average dose of GN for the twelve active cells was 50.7 ± 0.9 µg/cm^2. Due to rapid loss by evaporation the recoveries of applied GN were low (14.1 ± 0.4%). 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 normalization 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 2.38 ± 0.16 µg/cm^2 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

Compartment	GN (µg/cm^2)	GN (% applied dose)
Wipe	3.16 ± 0.10	6.23 ± 0.16
Donor chamber	0.935 ± 0.112	1.84 ± 0.22
Strip 1	0.222 ± 0.028	0.440 ± 0.056
Strips 2-3	0.248 ± 0.030	0.487 ± 0.058
Strips 4-6	0.140 ± 0.025	0.274± 0.048
Strips 7-10	0.064 ± 0.012	0.124 ± 0.022
Epidermis	0.210 ± 0.025	0.416 ± 0.050
Filter paper	0.285 ± 0.022	0.560 ± 0.040
Permeated	1.90 ± 0.15	3.74 ± 0.30
Overall recovery	7.15 ± 0.3	14.1 ± 0.4

Table 4: Absorption of the reference permeant (BA)

Reference Permeant	Experimental Results	[1]Multi-centre study Results	[1]Multi-centre study Highest Absorption Rates
BA (Benzoic Acid)	32.4 µg/cm^2 h	2.87 to 38.20 µg/cm^2h	30 to 32 µg/cm^2h

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 wipe.

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/cm^2h) fell in the range of the permeation rates reported in the multicenter study (van de Sandt *et al.*,).[6]

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 membrane 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 perfumery the maximum skin level of GN in a formulae that go into fine fragrances has been reported to be 0.24% (IFRA, 2004)[5], assuming use of the fragrance oil at levels up to 20% in the final product. The 97.5%ile use level in formulae for use in cosmetics in general has been reported to be 0.7% (IFRA, 2004)[5], 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.

Finally, validity of the test systems was confirmed by comparing the experimental absorption of the reference permeant with the measurement values in a multicenter study (van de Sandt et al., 2004).[6] As presented in Table 4, the experimental BA permeation (32.4 µg/cm^2h) fell in the range of the permeation rates reported in an *in-vitro* skin absorption multicenter study (van de Sandt *et al.*, 2004).[6] Our experimental results were in the high end of the absorption rates reported in multicenter study because, in the multicenter study, the thinnest (0.3-0.5 mm) dermatomed membranes resulted in high absorption and the epidermal membranes used this study exhibited the barrier properties similar to the dermatomed membranes used in the multicenter study (van de Sandt *et al.*, 2004).[6]

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