

OPINION OF THE SCIENTIFIC COMMITTEE ON COSMETIC PRODUCTS AND NON-FOOD
PRODUCTS INTENDED FOR CONSUMERS

CONCERNING

HEXAHYDRO-HEXAMETHYL-CYCLOPENTA (γ)-2-BENZOPYRAN
(HHCB)

adopted by the SCCNFP during the 21st plenary meeting
of 17 September 2002

1. Terms of Reference

The SCCNFP was requested to give an opinion concerning the safety for use in cosmetic products of Hexahydro-hexamethyl-cyclopenta (γ)-2-benzopyran and the potential need for any restrictions or conditions for its use.

Basing its assessment only on review of the cosmetic use of HHCB and considering the information provided in the 6 submissions previously made by COLIPA *, the SCCNFP has issued on 24 October 2000 an opinion requesting :

- additional information on the purity of the substance and its possible isomers,
- a new in vitro penetration study in accordance with the SCCNFP Notes of Guidance (SCCNFP/0119/99) in order to complement its information related to the percutaneous absorption of HHCB,
- an in vitro photo-toxicity study in accordance with the validated 3T3 Neutral Red Uptake Assay.

In submission VII, COLIPA has provided a set of information which is considered by the SCCNFP.

* *Submission n° 1 of December 1996, Submission n° 2 of April 1997, Submission n° 3 of July 1997, Submission n° 4 of July 1998, Submission n° 5 of March 1999 and Submission n° 6 of February 2000.*

2. Toxicological Evaluation and Characterisation

2.1. Purity of the Substance

According to the additional information which has been provided, HHCB is mainly composed by :

- 1,3,4,6,7,8 hexahydro-4,6,6,7,8,8-hexamethyl-cyclopenta- γ -2-benzopyran, CAS number 1222-05-5 corresponding to 74-76 % of the substance.

The following isomers were identified :

- 1,3,4,6,7,8-hexahydro-4,6,6,8-tetramethyl-(6 or 8)-ethylcyclopenta- γ -2-benzopyran, CAS numbers 78448-48-3 and 78448-49-4 corresponding to 6-10% of the substance.
- 1,3,4,7,8,9-hexahydro-4,7,7,8,9,9-hexamethyl-cyclopenta[H]-2-benzopyran, CAS number 114109-63-6 corresponding to 5-8% of the substance.
- 1,2,4,7,8,9-hexahydro-1,7,7,8,9,9-hexamethyl-cyclopenta[F]-2-benzopyran, CAS number 114109-62-5 corresponding to 6-8% of the substance.

The purity of the total substance is 95%, the impurities being :

- 1,1,2,3,3-pentamethyl-5-t-pentylindan, < 1% w/w
- 1,1,2,3-tetramethyl-5-t-butyl-3-ethylindan, < 1% w/w

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- β , 1,1,2,3,3-hexamethylindan-5-ethanol, CAS number 1217-08-9, < 1% w/w
- 5-t-butyl-1,1,2,3,3-pentamethylindan, CAS number 66553-13-7, < 1% w/w
- 1,1,2,3,3-pentamethylindan, CAS number 1203-17-4, < 1% w/w

2.2. Percutaneous Penetration

Previous results (Submissions I to VI)

In Animals

Fresh dorsal skin from rat maintained at 32° C was placed on a receptor liquid (flow rate = 1.5 ml/hour). 0.1 % and 0.5 % dose solutions of ¹⁴C-HHCB (15 µg/cm² or 78 µg/cm²) in solution in ethanol, diethyl phthalate (75/25) were applied to the skin occluded or unoccluded. At the end of experiment, skin was collected to be washed then digested in methanolic sodium hydroxide 1-2 hours at 70°C. Total radioactivity was measured by liquid scintillation counting.

<i>Occlusive system</i>	<i>Recovered in the receptor fluid at 24 hours</i> <i>(% of the quantity applied)</i>	<i>Recovered in the receptor fluid at 48 hours</i> <i>(% of the quantity applied)</i>	<i>Skin residue 24 hours</i> <i>(% of the quantity applied)</i>
Occluded (n = 6)	5.55 ± 3.92	17	46.58 ± 13.62
Unoccluded (n = 8)	0.07 ± 0.03	3	66.22 ± 7.65

These results demonstrated that HHCB crossed slowly the skin barrier (5% of the dose applied) even after occlusion and 48 hours were necessary for HHCB to cross skin barrier to a large extent (17 % dose applied). The occlusion process increased for about 5 fold the skin permeability.

Ref. : 55

In Humans

¹⁴C-HHCB was dissolved in ethanol/water (7/3) vehicle (2.4 mg/ml) and applied at the rate of 10.9 µg/cm² to a 100 cm² area of the skin on the back of each of three male subjects. The treated area was covered with gauze dressings after 30 min. After 6 hours the dressings were removed and the treated area was washed with 70 % aqueous ethanol soaked swabs. An area of 6.25 cm² was stripped with adhesive tape. The treated area was reoccluded with fresh dressings as necessary up to 120 h after which the dressings were removed and skin stripping was performed again on a different area of skin. Samples of whole-blood and excreta were taken during the 5 day study period.

The skin treated site was re-covered with fresh dressings up to 120 hours after compound application. 120 hours later dressings were taken off and another skin area of 6.25 cm² was stripped to determine the remaining total radioactivity in the stratum corneum. Samples of blood and excreta (urine and faeces) were collected during the five-day period.

Results : Recovery in excreta was below the limits of accurate detection (< 0.1 % AR). Concentration in whole blood and plasma was also below the limits of accurate measurement at all sampling times.

The mean total recovery from excreta, dressings, swabs and skin strips was 76.22 % AR with a range of 72.60-79.45 % AR.

Most of the radioactivity (56.33 % AR) was recovered from the dressings and skin washes 6 hours after application. A further 18.9 % AR was detected in dressings at later time suggesting that some radioactivity remained in the skin after washing. 10.9 % AR of the applied radioactive compound was found in the strips 6 hours after application ; only 0.24 % AR was found in the strips 120 hours after application.

Ref. : 49

In vitro Human Skin Penetration Study (Submission VII)

An additional in vitro study of the human skin penetration rate and distribution of radio-labelled HHCB following application under non occlusive conditions has been submitted.

- The applied dose was 20 $\mu\text{l}/\text{cm}^2$ of the HHCB 1 % solution in ethanol (corresponding to 200 $\mu\text{g}/\text{cm}^2$ HHCB).
- 200 μl samples were taken from each receptor chamber at 1, 2, 6, 12 and 24 hours then placed into scintillation fluid and analysed for ^{14}C using liquid scintillation counting (LSC)
- Following the last receptor phase sample, HHCB remaining on the surface of each of the epidermal membrane was removed with a cotton bud which was then extracted in methanol for analysis. Each epidermal membrane was tape stripped 10 times (D-square). The strips were grouped (strip 1, strips 2-3, strips 4-6 and strips 7-10) and then solubilized (Optisolve) and analysed. The remaining samples of skin were solubilized individually (Optisolve) prior to sampling and analysing.

The diffusion cell donor samples were wiped to remove sealing grease. Wipes were extracted (tetrahydrofuran). The diffusion cell donor samples were then washed in methanol. The filter paper supports were extracted into methanol.

- Evaporation loss of HHCB in ethanol was assessed under the same experimental conditions, using polytetrafluoroethylene sheet (PTFE) dosed with 20 $\mu\text{l}/\text{cm}^2$ of the 1 % HHCB solution in ethanol.
- The amounts of HHCB per unit of area ($\mu\text{g}/\text{cm}^2$) in the receptor phase and various compartments of the skin and diffusion cells were determined and then the percents of the applied dose of HHCB on those compartments were calculated.

Radiolabelled HHCB was prepared as a 1 % (w/v) solution in ethanol (96 % v/v) ; radiolabel content and uniformity of the dispersion were controlled prior the experimentation.

- The test membranes, isolated epidermis, used for the study were proceeding from human female breast and abdominal skin, obtained from 3 donors after cosmetic surgery and stored at -20°C . After removing the subcutaneous fat, individual portions of skin were immersed in

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water at 60°C for 50 seconds and then the upper layer (comprising stratum corneum and epidermis) was removed from the underlying dermis. The membranes were then stored at – 20°C until use.

On the day of use epidermal membranes were taken up onto 25 mm diameter filter paper support and then mounted onto diffusion cells. Twelve active dosed cells were prepared plus two control cells. Membrane integrity was then assessed by measuring the penetration rate of tritiated water over a period of 1 hour.

Results : Following 24 hour exposure the distribution of HHCB in all compartments (mean \pm SE) is summarized in the following tables :

HHCB dosed cells ($\mu\text{g}/\text{cm}^2$)

Nber of cells = 12	1 surface wipe	2 strip 1	3 strip 2-3	4 strip 4-6	5 strip 7-10	6 remaining skin	7 receptor phase	8 donor chamber wash	9 filter paper	10 total recovered
Mean	116	4.29	4.08	2.13	1.15	9.04	0.795	45.8	0.490	184
SD	13	1.73	2.42	1.16	0.63	3.93	0.398	9.5	0.216	6
SE	4	0.50	0.70	0.33	0.18	1.13	0.115	2.8	0.062	2

HHCB dosed cells (% applied dose)

Mean	58.2	2.15	2.04	1.07	0.578	4.52	0.397	22.9	0.245	92.1
SD	6.5	0.86	1.21	0.58	0.315	1.96	0.197	4.8	0.108	2.7
SE	1.9	0.25	0.35	0.17	0.091	0.57	0.057	1.4	0.031	0.8

According to these results :

- the exact amount of HHCB applied to the skin was $199.8 \pm 0.7 \mu\text{g}/\text{cm}^2$,
- the total recovery of HHCB is $92.1 \pm 0.8 \%$ of the applied dose (column 10). It results from the study performed with the PTFE sheets that a small quantity of HHCB evaporates in 24 hours, allowing to consider that the overall recovery of HHCB moves to a value closer to 95 % ,
- the majority of the applied doses found in the surface wipe (column 1) and donor wipe chamber wash (column 8), corresponding to $81.3 \pm 2.1 \%$ of the applied dose,
- a significant amount of HHCB is found in all stripped layers of the stratum corneum (column 2, 3, 4, 5) corresponding to $5.83 \pm 0.82 \%$ of the applied dose (It can be observed that the distribution within the layers strongly decreases with skin depth),
- human skin absorption of HHCB corresponding to the amounts found in receptor phase (column 7), filter paper (column 9) and remaining stratum corneum and epidermis (column 6) is $5.16 \pm 0.59 \%$.

Using this figure, the percentage of absorption to be retained for the calculation of the safety margin should be 5.2 %.

Discussion

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This additional assay does not meet the requirements for practical and technical reasons and cannot be considered for the assessment of the safety margin :

- it does not conform to the SCCNFP Notes of Guidance,
- the use of pure ethanol as vehicle strongly affects the tissue and therefore the potential skin penetration of the substance.

Basing on all the information it seems therefore more convenient to consider the previous *in vivo* human study (ref. 49) which methodology remains more pertinent in spite of using a low concentration of HHCB.

According to this study it can be retained that the skin permeability of ¹⁴C-HHCB in human is negligible and below the limits of accurate measurement, i.e. less than 0.1% AR. Therefore 0.1% can be assumed as conservative absorption for the calculation of the safety margin.

2.3. Phototoxicity

A new Neutral Red Uptake toxicity assay had been submitted.

Test method	:	Neutral Red Uptake Phototoxicity Assay (OECD 432 (draft); EC/ B41; 19 th May 2000)
GLP	:	U.S. FDA 21 CFR 58; OECD
Test article	:	No. 99AC90; Sample D; colourless semi-viscous liquid; 05/04/99; (data on purity of sample not provided) (>95%)
Cell line	:	Balb/c 3T3 mouse fibroblast (ATCC)
Positive control	:	Chlorpromazine
Principle of the test method	:	Absorption of UV light by the chemical can result in a phototoxic response (cell damage). Comparison of cytotoxicity of the chemical when tested in the presence and in the absence of UV exposure.
UV radiation	:	Dermalight SOL 3 solar simulator, equipped with UVA H1 filter (320-440 nm). Delivery of 1.7 ± 0.1 mW/cm ² of UVA energy resulting in a dose of 1 J/cm ² per 10 min
Replicate	:	4-6 replicate wells; duplicate plates
Doses	:	6-8 decreasing concentrations
Results (PIF)	:	Test Article; 1.32; 1.12; (NEGATIVE)* Positive control: 13.620; 18.359 (POSITIVE)
Laboratory	:	Institute for <i>In Vitro</i> Science, Inc, USA
Date	:	20 th Sept. 2001
Conclusion	:	HHCB is not phototoxic when tested with the Neutral Red Uptake Phototoxicity Assay on Balb/c 3T3 mouse fibroblast cells treated <i>in vitro</i>

* A chemical is considered phototoxic is the PIF > 5.0 (Photo Irritancy Factor)

2.4. New Calculation of the Safety Margin

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In a previous submission COLIPA provided a table corresponding to the estimated total consumer exposure to HHCB through fragranced cosmetic products, considering that the range of cosmetic products selected covers all those that are likely to be used in any one weekly period.

Calculation of Exposure to HHCB in Cosmetic Products, the retention factors of bath products, shower gel, toilet soap and hair spray are corrected according to the “Notes of guidance for testing of cosmetic products for their safety evaluation (third revision) – Annex 5” :

Type of cosmetic product	Application quantity in grams per application	Application frequency per day	Retention factor (%)	Fragrance compound in product (%)	HHCB in fragrance compound (%)	HHCB in product (%)	Exposure to HHCB (mg/day)	Exposure to HHCB for 60 kg person (µg/kg/day)
Body lotion ⁽¹⁾	8	0.71	100	0.4	30	0.120	6.826	113.6
Face cream ⁽²⁾	0.8	2	100	0.3	30	0.090	1.440	24.0
Eau de toilette ⁽³⁾	0.75	1	100	8.0	30	2.400	18.000	300.0
Fragrance cream ⁽¹⁾	5	0.29	100	4.0	30	1.200	17.400	290.0
Anti-perspirant /deodorant	0.5	1	100	1.0	30	0.300	1.500	25.0
Shampoo	8	1	1	0.5	30	0.150	0.120	2.0
Bath products ⁽⁴⁾	17	0.29	1	2.0	30	0.600	0.30	5.0
Shower gel ⁽⁴⁾	5	1.07	10	1.2	30	0.360	1.930	32.0
Toilet soap	0.8	6	10	1.5	30	0.450	2.160	36.0
Hair spray	5	2	10	0.5	30	0.150	1.500	25.0
						Total	51.176	852.6

- (1) Assumes use of conventional body lotion 5 times per week and a fragranced cream twice per week.
- (2) Including make up and foundation.
- (3) Including perfume and after shave, but these three products are not used concurrently. The quantity used is inversely proportional to the fragrance concentration so these values include all hydroalcoholic products.
- (4) Assumes use of bath products twice per week and an average use of shower gel 1.5 times per day, 5 times per week.

Based on such exposure the safety margin can be calculated :

Maximum daily exposure to HHCB	I (mg)	=	51.176 mg
Maximum absorption through the skin	A (%)	=	0.1 %
Maxim dermal absorption per day	I x A	=	0.0512 mg
Typical body weight of human		=	60 kg
Systemic exposure dose (SED)	I x A / 60 kg	=	0.85 µg/kg
No observed adverse effect level (mg/kg)	NOAEL	=	50 mg/kg

Margin of Safety	NOAEL x 1000 / SED =	58823
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2.5. Opinion

On review of the information currently available, the SCCNFP is of the opinion that HHCB can be safely used as a fragrance ingredient in cosmetic products without any restriction for its use.

The above has been formulated only as a review of the cosmetic use of HHCB. For a full safety assessment of HHCB, it is necessary to consider other sources of consumer exposure from non-food products e.g. laundry products.

2.6. References

90. Harbell et al. Neutral Red Uptake Phototoxicity Assay in Balb/c 3T3 Mouse fibroblasts. Institute for in Vitro Science. September 20, 2001.
91. M. Green et al. In vitro human skin penetration of radiolabelled fragrance material HHCB. An-eX Analytical Services. September 10, 2001.