Human and Environmental Risk Assessment

on Ingredients of Household Cleaning Products

HHCB

(1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-γ-2-benzopyran and related isomers)

(CAS 1222-05-5)

Version 2.0

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1 EXECUTIVE SUMMARY

HHCB (1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-gamma-2-benzopyran and related isomers) is a member of a group of substances used in fragrances and known collectively as the polycyclic musks. Because it is a highly viscous liquid, it is sold fluidised as an approximately 65% solution.

HHCB is used to make a fragrance long lasting and have a positive technical effect on its balance bringing the initial and residual smell into harmony.

HHCB is produced in one plant in the UK in an annual volume of 1000 to 5000 tonnes and is transported to Ireland for dilution to the commercial product.

It is used as an ingredient in commercial preparations intended to be used as fragrances in a wide variety of consumer products such as perfumes, cosmetics, household and laundry cleaning products and air fresheners. These commercial preparations are not sold retail. The level of HHCB in such preparations is typically at a level of several percent. The principal exposure to HHCB from household products can be considered to be via the skin.

The relative volume of use in household products versus perfumes, cosmetics, etc. is not known although the majority can be assumed to be used in household products based on the relative volume of sales of such products.

Environmental

To be completed.

Human health

HHCB has a low acute toxicity either by the oral or dermal route (LD_{50} values >3000 mg/kg). Inhalation exposure has been estimated to be negligible relative to dermal.

HHCB is not a skin or eye irritant and shows no phototoxicity potential on humans at concentrations significantly higher than would be encountered from the use of fragranced consumer products. There is no significant evidence either from animal or human studies of potential for dermal sensitisation. HHCB shows no photosensitisation potential on humans at concentrations significantly higher than would be encountered from the use of fragranced consumer products.

In a 90-day study in rats, there were no adverse effects at the highest dose tested, 150 mg/kg bw/day.

There were no indications of effects on fertility or the developing foetus at levels as high as 50 mg/kg bw/day.

There were no effects on rat pups exposed via the milk during nursing to levels of HHCB over 100 times the maximum level found in human milk samples.

HHCB is a non-genotoxic substance. The mutagenicity data and the repeated dose studies with HHCB do not indicate a concern with regard to carcinogenicity nor does HHCB possess any structural features that would raise a concern.

In the unlikely event of maximum exposures from direct and indirect skin contact as well as from the oral route via dishware residues, the estimated exposure to HHCB from its use in household cleaning products of 0.07 µg/kg bw/day. Comparison of this exposure to the NOAEL indicates a margin of safety of at least 350,000 and supports the conclusion that there

is no significant risk to human health from exposure to HHCB as used in household cleaning products.

2 CONTENTS

3 SUBSTANCE CHARACTERISATION

3.1 CAS NO and grouping information

HHCB (1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-γ-2-benzopyran and related isomers) (CAS 1222-05-5) is a widely used ingredient of fragrance formulations used in soaps, detergents and other cleaning products as well as in cosmetics and fine perfumes.

Sum of isomers (>95%) with typical composition:

1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl-cyclopenta-γ-2-benzopyran,

74-76%, CAS# 1222-05-5;

1,3,4,6,7,8-hexahydro-4,6,6,8-tetramethyl-(6 or 8)-ethylcyclopenta-γ-2-benzopyran, 6-

10%, CAS#s 78448-48-3 and 78448-49-4;

1,3,4,7,8,9-hexahydro-4,7,7,8,9,9-hexamethyl-cyclopenta[H]-2-benzopyran,

5-8%, CAS# 114109-63-6;

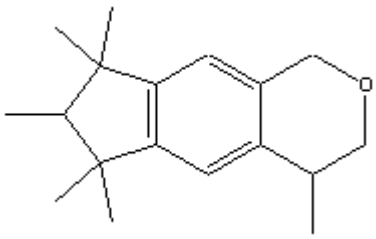
1,2,4,7,8,9-hexahydro-1,7,7,8,9,9-hexamethyl-cyclopenta[F]-2-benzopyran,

6-8%, CAS# 114109-62-5.

HHCB is also known and marketed as Abbalide, Chromanolide, Galaxolide, HHCB, and Pearlide by different manufacturers.

3.2 Chemical structure and composition

Molecular description



HHCB (principal isomer)

Macro-molecular description (Physical State/Particle size)

HHCB is a mixture of isomers and as such, is a viscous liquid at room temperature. To facilitate handling of the material, it is fluidised with an odour neutral diluent. It is sold and used as an approximate 65% solution in the diluent. The most common diluents used are diethyl phthalate (DEP), benzyl benzoate (BB) and isopropyl myristate (IPM).

Molecular formula/weight

$C_{18}H_{26}O$ – MW 258.41

Melting point

-10 to 0 ^{o}C

Boiling point

The boiling point as recorded in the distillation of HHCB in the manufacturing plant is 160 ^{o}C at 4 hPa. This translates mathematically to 330 ^{o}C at 760 mm Hg.

Vapour pressure at 25° C

0.000727 hPa at 25 ^{o}C

Octanol-water partition coefficient

5.9 at 25 ^{o}C

Water solubility

1.75 mg/l at 25 ^{o}C

Density

0.99 - 1.015 g/cm3 at 20 ^{o}C

Henry's constant

1.059e-4 atm m3/mole (calculated)

3.3 Manufacturing & production/volume

The European Union has currently only one manufacturer of HHCB, IFF, in an annual volume of 1000 to 5000 tonnes (2001). Other companies have terminated their production. BBA (Bush Boake Allen Inc.) was purchased by IFF in November of 2000. BBA has also terminated production of HHCB as of early 2001.

Fragrance companies receive HHCB from suppliers in and outside of the EU market or through brokers importing the substance. Fragrance formulations containing HHCB are supplied by fragrance companies to their customers worldwide for incorporation into the final product. The end products are fragranced household products and cosmetics that are sold in the EU market as well as exported to countries worldwide.

A brief summary of the manufacture/market lifecycle of HHCB is as follows: 1) HHCB is manufactured by organic synthesis. Synthesized HHCB is a very viscous product. 2) For ease of use it is commonly marketed and used as an approximately 65% solution in a neutral diluent. This grade of HHCB is supplied to fragrance compounding houses as an ingredient for use in blending. 3) Fragrance compounding facilities produce fragrances by blending fluidised HHCB with a variety of other fragrance ingredients. 4) These fragrance formulations are used by product manufacturers in various consumer products. The HHCB concentration in the final product is a very small fraction. 5) The end product is available for use by consumers

worldwide (e.g. detergents, household cleaning products, and cosmetics). Since the synthesis and patenting of HHCB in the 1960s, HHCB has been used as an ingredient in fragrances with a musky scent.

HHCB Manufacturing process: HHCB is manufactured by a reaction of iso-amylene and alpha methyl styrene followed by Friedel Crafts reaction with propylene oxide and a further reaction with para-formaldehyde. These syntheses are highly automated and occur as either continuous or batch reactions in a closed system. The end product is a result of further distillation and purification. Manufactured HHCB is highly viscous and is therefore, fluidised by a batch blending process and sold as an approximate 65% solution. Dosage by weighing is automated where possible into closed stationary bulk tanks with local ventilation.

3.4 Use applications summary

HHCB is used as an ingredient in commercial preparations intended to be used to fragrance a wide variety of consumer products such as perfumes, cosmetics, household and laundry cleaning products and air fresheners. These commercial preparations are not sold retail. The level of HHCB in such preparations is typically at a level of several percent. The principal exposure to from household products can be considered to be via the skin. The reasonable maximum use levels in household cleaning products ranges from 0.02 to 0.9% (see Table 1). This reports covers only the uses in household cleaning products.

4 ENVIRONMENTAL ASSESSMENT

4.1 Environmental exposure assessment

4.1.1 Environmental fate

Biotic and abiotic degradability

a) Ready test

b) Biodegradation in river water

c) Anaerobic degradation

c) Biodegradation in soil

d) Hydrolysis

e) Photolysis

4.1.2 Removal

Removal in sewage treatment

a) % degraded

b) % to water

c) % to sludge

% to air

4.1.3 Monitoring Studies

a) Water

b) Air

c) Soil

d) Sewage

4.1.4 PEC Calculations

a) PEC Water
b) PEC Soil:
c) PEC Sediment
d) PEC STP
e) Concentration in dry sewage sludge

4.2 Environmental effects assessment

4.2.1 Toxicity

4.2.1.1 Ecotoxicity – Aquatic: acute test results

a) Algae EC50

b) Invertebrate IC50

c) Fish LC50

d) Other EC*50*

4.2.1.2 Ecotoxicity – Aquatic: chronic test results

a) Algae NOEC

b) Invertebrate NOEC

c) Fish NOEC

d) Other NOEC including mesocosm data

4.2.1.3 Terrestrial – acute test results

a) Plants LC50

b) Earthworms LC50

c) Micro-organisms LC50

d) Other LC50

4.2.1.4 Terrestrial – chronic test results

a) Plants NOEC

b) Earthworms NOEC

c) Micro-organisms NOEC

d) Other NOEC

4.2.1.5 Micro-organisms e.g. in Wastewater Treatment

4.2.2 PNEC calculations

a) PNEC water

b) PNEC sediment

c) PNEC soil

d) PNEC

4.3 Environmental risk characterisation

a) RCR Water

b) RCR Soil

c) RCR Sediment

d) RCR STP

4.4 DISCUSION AND CONCLUSIONS

5 Human Health Assessment

5.1 Consumer Exposure

5.1.1 Product Types

In line with the objectives of the HERA initiative, this human health assessment focuses on the use of HHCB (1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-γ-2-benzopyran and related isomers) as an ingredient of fragrance oils used in household cleaning products. HHCB is also used as an ingredient in fragrances used in perfumes, cosmetics and other consumer products. This report covers only exposures resulting from its use in household products. The other uses have been reviewed by the SCCNFP (SCCNFP, 2002), which concluded "that HHCB can be safely used as a fragrance ingredient in cosmetic products without any restriction for its use."

Both the fragrance manufacturing industry and the consumer product industry were surveyed by the International Fragrance Association (IFRA, 2002) to determine the use levels of fragrance oils in product types and the levels of HHCB that are used to formulate these oils in the EU and other geographic locations. Because most household cleaning products are fragranced and HHCB is a common fragrance ingredient, it is found in some products in each of the HERA product categories (Table 1) (IFRA, 2002).

Table 1. Use levels of HHCB in household cleaning products. Results of a survey including data from manufacturers of fragrances as well as finished products (IFRA, 2002)

Product category	Median use level of fragrance oil in product in %	97.5 percentile use level of HHCB [a]	Level of HHCB in product
Laundry regular powder	0.33	15%	0.05%
Laundry liquid	0.80	15%	0.12%
Laundry compact (tabs)	0.33	15%	0.05%
Laundry compact (powder and other)	0.28	15%	0.04%
Laundry liquid concentrate	0.85	15%	0.13%
Fabric softener (conditioner)	0.43	15%	0.06%
Fabric softener concentrate	0.80	15%	0.12%
Laundry additive, powder bleach	0.20	15%	0.03%
Laundry additive, liquid bleach	0.20	15%	0.03%
Laundry additive, tablet	0.30	15%	0.05%
Hand dishwashing liquid	0.23	15%	0.04%
Hand dishwashing liquid concentrate	0.45	15%	0.07%
Machine dishwashing powder	0.15	15%	0.02%
Machine dishwashing liquid	0.15	15%	0.02%
Machine dishwashing tablet	0.15	15%	0.02%
Surface cleaner liquid	0.60	15%	0.09%
Surface cleaner powder	0.25	15%	0.04%
Surface cleaner gel	0.75	15%	0.11%

Product category	Median use level of fragrance oil in product in %	97.5 percentile use level of HHCB [a]	Level of HHCB in product
Surface cleaner spray	0.13	15%	0.02%
Toilet cleaner powder	0.30	15%	0.05%
Toilet cleaner liquid	0.35	15%	0.05%
Toilet cleaner gel (concentrate)	0.38	15%	0.06%
Toilet cleaner tablet	0.30	15%	0.05%
Toilet rim block or gel	6.0	15%	0.9%

[a]97.5 percentile use level of HHCB in fragrance oils used in household and detergent products

5.1.2 Consumer Contact Scenarios

Using any and all of the above products results in some exposure to HHCB, either dermally through direct contact, orally as a result of residues in drinking water or on dishes, and by inhalation of aerosols from cleaning sprays. In addition, since HHCB is used for its fragrance properties, some inhalation will result from evaporation from all of the listed products if they are fragranced, however, because of the very low volatility of HHCB and its low level of use, inhalation is not a significant route of exposure when compared to dermal exposure.

All of these potential exposures are addressed below with explanations as to how the estimates were made. However, it is unreasonable to assume a consumer would use all of the listed products since many are redundant. For example, a consumer when doing hand washing of laundry would use either regular powder or a liquid detergent but not both. For this reason, only one of the products in any given box shown in Table 1 is used for exposure estimation; the worst case is selected.

Furthermore, for some uses, the exposures are negligible relative to other exposures and, while discussed below, no calculations are made.

5.1.3 Consumer Exposure Estimates

For the estimates of consumer exposure, the consumer exposure models given in the HERA guidance document are used along with the data presented in the Table of Habits and Practices for Consumer Products in Western Europe, which was issued by the European Soap and Detergent Industry Association, AISE (AISE/HERA, 2002). This table presents use data for cleaning products in grams/task, use frequency, duration of task and other intended uses. While minimum, maximum and typical use frequencies and amounts are given; only the maximum figures are used for the exposure estimations with the understanding that further refinement will be possible if necessary. In some cases, it is necessary to make additional assumptions, where so, these are described.

Finally, a total exposure is calculated even though it is highly unlikely (even impossible) that any consumer would (or could) use products from all of the categories in maximum amount, at maximum frequencies and with each of these products being fragranced with fragrance oils containing HHCB at the 97.5 percentile use level.

5.1.3.1 Direct skin contact from hand-washed laundry

Hand-washed laundry is a common consumer habit. During this procedure, the HHCB-containing laundry solution with an estimated product concentration of 10 mg/ml comes in direct contact with the skin of hands and forearms. A hand-washing task typically takes 10 minutes (Table of Habits and Practices - AISE/HERA, 2002). This table also reports a maximum frequency of 18 times per week (3 times/day) when using laundry powder, which seems highly exaggerated but nevertheless is used here as a worst case scenario. The table gives a lower frequency of hand washing with laundry liquid of 10 times per week (1.43 times/day), which still seems exaggerated. Because the use level of HHCB is different in powder (0.05%) from that in liquid (0.12%) both scenarios are calculated here.

The exposure to HHCB is estimated according to the following algorithm from the HERA guidance document.

$$\mathbf{Exp_{sys} = F_1 \times C \times Kp \times t \times S_{der} \times n / BW}$$

For this exposure estimate, the terms are defined with following values for the calculation considering a worst-case scenario:

F_1	percentage weight fraction of substance in product	**0.05%** (0.0005) or **0.12%** (0.0012) (Table 1)
C	product concentration in mg/ml:	**10 mg/ml** (AISE/HERA, 2002)
Kp	dermal penetration coefficient	**4.29 x 10^{-5} cm/h*** (Green and Brain, 2001)
t	duration of exposure or contact	**10 min (0.167h)** (AISE/HERA, 2002)
S_{der}	surface area of exposed skin	**1980cm^2** (TGD, 1996)
n	product use frequency (tasks per day)	**3** or **1.43** (AISE/HERA, 2002)
BW	body weight	**60 kg** (TGD, 1996)

* The dermal penetration coefficient was calculated from the dermal flux (10.3 µg/cm^2) which was determined in an *in vitro* dermal penetration (Green and Brain, 2001) according to the following algorithm: Kp = dermal flux/(exposure time x concentration of test solution); Kp = (0.0103 mg/cm^2)/(24h x 10 mg/cm^3) = 4.29 x 10^{-5} cm/h

For powder use:

Exp_{sys} = [0.0005 x (10 mg/ml) x (4.29 x 10^{-5} cm/h) x (0.167h) x 3 x (1980 cm²)] / 60 kg=

0.0035 µg/kg bw/day

For liquid use:

Exp_{sys} = [0.0012 x (10 mg/ml) x (4.29 x 10^{-5} cm/h) x (0.167h) x 1.43 x (1980 cm²)] / 60 kg=

0.0040 µg/kg bw/day

The more conservative figure is used in calculated aggragate total exposure.

5.1.3.2 Direct skin contact from laundry detergent

Filling laundry detergent into the dispenser of the washing machine involves only a very short direct skin contact with the neat material. Due to the short contact time and the very small skin contact area, the dermal exposure to HHCB from this use is considered insignificant relative to other exposures.

5.1.3.3 Direct skin contact from pre-treatment of clothes

Consumers typically spot-treat clothing stains by hand using either a detergent paste (i.e. water/laundry powder = 1:1) or a laundry liquid, which is applied undiluted (i.e. concentration = 1000 mg/ml) directly on the garment. In this exposure scenario, only the skin surface of the hand (~ 840 cm^2) is exposed.

The exposure to HHCB is estimated according to the same algorithm from the HERA guidance document as is used in 5.1.3.1 above using the liquid detergent since this is the highest concentration of HHCB.

F_1	percentage weight fraction of substance in product	**0.12%** (laundry liquid; 0.0012) (Table 1)
C	product concentration in mg/ml:	**1000 mg/ml** (100%)
Kp	dermal penetration coefficient	**4.29 x 10^{-5} cm/h** (Green and Brain, 2001)
t	duration of exposure or contact	**10 min (0.167h)** (AISE/HERA, 2002)
S_{der}	surface area of exposed skin	**840cm^2** (TGD, 1996)
n	product use frequency (tasks per day)	**0.5** (AISE/HERA, 2002)
BW	body weight	**60 kg** (TGD, 1996)

Exp_{sys} = [0.0012 x (1000 mg/ml) x (4.29 x 10^{-5} cm/h) x (0.167h) x (840 cm^2) x 0.5]/ 60 kg=
0.060 µg/kg bw/day

This exposure estimate is very conservative in that it does not recognize use of water to dilute the detergent, a common practice and the fact that only a fraction of the two hands' surface skin will actually be exposed.

5.1.3.4 Direct skin contact from hand dishwashing

The determination of HHCB exposure from hand dishwashing also uses the algorithm discussed in chapter 5.1.3.1 is used to calculate the dermal exposure to HHCB from hand dishwashing. The following assumptions have been made to address a reasonable worst-case scenario:

F_1	percentage weight fraction of substance in product	**0.04%** (0.0004) (Table 1)
C	product concentration in mg/ml:	**2 mg/ml** (AISE/HERA, 2002)
Kp	dermal penetration coefficient	**4.29 x 10^{-5} cm/h** (Green and Brain, 2001)
t	duration of exposure or contact	**45 min** (0.75h) (AISE/HERA, 2002)
S_{der}	surface area of exposed skin	**1980 cm^2** (TGD,1996)
n	product use frequency (tasks per day)	**3** (AISE/HERA, 2002)
BW	body weight	**60 kg** (TGD, 1996)

Exp_{sys} = [0.0004 x (2 mg/ml) x (4.29 x 10^{-5} cm/h) x (0.75h) x (1980 cm²) x 3] / 60 kg=
0.0025 µg/kg bw/day

5.1.3.5 Direct skin contact from hard surface cleaning

During this procedure, the HHCB-containing hard surface cleaning solution comes in direct contact with the skin of the hands. A hard surface-cleaning task takes at maximum 20 minutes (AISE/HERA, 2002). The exposure to HHCB is estimated according to the following algorithm from the HERA guidance document:

$$\mathbf{Exp_{sys} = F_1 \times C \times Kp \times t \times S_{der} \times n / BW}$$

For this exposure estimate, the terms are defined with following values for the calculation considering a worst-case scenario:

F_1	percentage weight fraction of substance in product	**0.09%** (0.0009) (Table 1)
C	product concentration in mg/ml:	**12 mg/ml** (AISE/HERA, 2002)
Kp	dermal penetration coefficient	**4.29 x 10^{-5} cm/h** (Green and Brain, 2001)
t	duration of exposure or contact	**20 min (0.334h)** (AISE/HERA, 2002)
S_{der}	surface area of exposed skin	**840cm²** (TGD (1996))
n	product use frequency (tasks per day)	**1** (AISE/HERA, 2002)
BW	body weight	**60 kg** (TGD, 1996)

Exp_{sys} = [0.0009 x (12 mg/ml) x (4.29 x 10^{-5} cm/h) x (0.334h) x 1 x (840 cm²)] / 60 kg=
0.0021 µg/kg bw/day

5.1.3.6 Indirect skin contact from wearing clothes

Residues of components of laundry detergents may remain on textiles after washing and can transfer from the textile to the skin. There are no data available showing how much HHCB is deposited on the fabric following a wash process. If 1 kg of clothes retains 600 ml rinse water (Henkel KGaA, 2002) and that rinse water contains 2.5 % (ZVEI and IKW, 1999) of the detergent (and thus HHCB) used then the concentration of HHCB in that rinse water can be calculated:

600 ml x 10 mg/ml x 2.5% x 0.12% = 0.18 mg

If 100% is transferred to the 1 kg of fabric, then the concentration in the fabric will be 0.18 mg/kg. Given the fabric density of 10 mg/cm^2 (Procter & Gamble, 1996), it can be calculated that the HHCB is present at 1.8 x 10^{-6} mg/cm^2.

The following algorithm recommended in the HERA guidance document can then be used to estimate the dermal exposure to detergent residues in the fabric:

$$\mathbf{Exp_{sys} = F_1 \times C` \times S_{der} \times n \times F_2 \times F_3 \times F_4 / BW}$$

For the exposure estimate, the terms are defined with the following values for the calculation:

F_1	percentage weight fraction of substance in product	**1**
C`	product (HHCB) load:	**1.8 x 10^{-6} mg/cm^{2*}**
S_{der}	surface area of exposed skin	**17600 cm^2** (TGD (1996))
n	product use frequency (tasks per day)	**1 for 24 hr**
F_2	percent weight fraction transferred to skin	**1%** (0.01) (Vermeire et al., 1993)
F_3	percent weight fraction remaining on skin	**100%** (worst case)
F_4	percent weight fraction absorbed via skin	**5.2%** (0.052) for 24 hr (Green and Brain, 2001)
BW	body weight	**60 kg** (TGD, 1996)

$$\mathbf{Exp_{sys\ (indirect\ skin\ contact)}} = [(1.8 \times 10^{-6}\ mg/cm^2) \times (17{,}600\ cm^2) \times 0.01 \times 1 \times 0.052] \ / \ 60kg = \mathbf{2.7 \times 10^{-7}\ \mu g\ /kg\ bw\ day}$$

5.1.3.7 Inhalation of detergent dust during washing processes

Studies by van de Plassche et al., 1998 determined an average release of about 0.27 µg dust per cup of product (i.e. laundry powder) is used for machine laundering. HHCB is present in laundry powder detergents at a level of 0.05% (or 1.35 x 10^{-4} µg HHCB/use). Taking the worst-case assumption that 100% of released dust is inhaled and washing of laundry occurs 3 times daily, the exposure of an adult with an average body weight of 60 kg to HHCB is estimated to be:

$$\mathbf{Exp_{sys\ (inhalation\ of\ detergent\ dust)}} = [(1.35 \times 10^{-4}\ \mu g) \times 3] \ / \ 60\ kg = \mathbf{6.75 \times 10^{-6}\ \mu g/kg\ bw/day}$$

5.1.3.8 Inhalation of aerosols from cleaning sprays

HHCB is also present in surface cleaning sprays. The HERA guidance document specifies the algorithm to be used for calculation of consumers' worst-case exposure to HHCB-containing aerosols generated by the spray cleaner:

$$\mathbf{Exp_{sys} = F_1 \times C` \times Q_{inh} \times t \times n \times F_7 \times F_8 / BW}$$

F_1	percentage weight fraction of substance in product	***0.02%*** (0.0002) (Table 1)
C`	product concentration in air:	***0.35 mg/m³*** * (Procter & Gamble, 2001)
Q_{inh}	ventilation rate	***0.8 m³/h*** (TGD, 1996)
t	duration of exposure	***10 min*** (0.17h) (AISE/HERA, 2002)
n	product use frequency (tasks per day)	***1*** (AISE/HERA, 2002)
F_7	weight fraction of respirable particles	***100%***
F_8	weight fraction absorbed or bioavailable	***75%***; 075 (TGD, 1996)
BW	body weight	***60 kg*** (TGD, 1996)

$Exp_{sys\ (inhalation\ of\ aerosols)}$ = $(0.0002 \times (0.35\ mg/m^3) \times (0.8\ m^3/h) \times (0.17\ h) \times 0.75] / 60\ kg =$ **1.2×10^{-4} µg/kg bw/day**

* this value was obtained by experimental measurements of the concentration of aerosol particles smaller than 6.4 microns in size which are generated upon spraying with typical surface cleaning spray products

5.1.3.9 Oral Exposures to HHCB

Oral exposures to HHCB can arise from residues in food and drinking water as well as from residues on dishes. Analyses of fruits, vegetables and drinking water have not detected HHCB at the limits of detection. Levels in fish have been reported with a 90th percentile level in fish in the Berlin area of 1.5 mg/kg. Levels in drinking water (regional) are estimated using EUSES to be 0.034 µg/L. (These levels are a result of not only its use in household products but also from use in perfumes, cosmetics and other consumer products.) Assuming a daily intake of water of 2 L, a daily intake of fish of 0.115 kg (TGD, 1996) and 50% bioavailability, exposures from these sources a can be estimated as follows:

$Exp_{sys\ (oral\ via\ drinking\ water)}$ = $[(0.034\ µg/l) \times (2L) \times 0.5] / 60\ kg$ = **0.0006 µg/kg bw/day**

$Exp_{sys\ (oral\ via\ fish)}$ = $[(1.5\ mg/kg) \times (0.115\ kg) \times 0.5] / 60\ kg$ = **1.4 µg/kg bw/day**

The daily exposure to HHCB from eating with utensils and dishware that have been washed in dishwashing detergents can be estimated according to the following algorithm from the HERA guidance document:

$Exp_{sys} = F1 \times C` \times Ta' \times Sa / BW$

For this exposure estimate, the terms are defined with following values for the calculation considering a worst-case scenario:

F_1	percentage weight fraction of substance in product	***0.04%*** (0.0004); (Table 1)
C`	concentration of product in dish wash solution:	***2 mg/cm³***
$T_{a'}$	amount of water left on dishes after rinsing	***5.5 x 10⁻⁵ ml/cm²*** (Schmitz, 1973)

S_a	area of dishes in daily contact with food	***5400cm²*** (Official publication French legislation, 1990)
BW	body weight	***60*** **kg** (TGD, 1996)

$Exp_{sys\ (oral\ dish\ deposition)}$ = [0.0004 x (1 mg/cm^3) x (5.5 x 10^{-5} ml/cm^2) x (5400 cm^2)]/60 kg = **0.002 µg/kg bw/day**

5.1.3.10 Accidental or intentional overexposure

Accidental or intentional overexposure to HHCB may occur via household detergent products, which may contain up to 0.9% HHCB. Exposure may be oral as a result of ingestion of the product or dermally as a result of splashing onto the skin or into the eyes.

Ingestion is not likely to exceed 10 g of a household product. Given the maximum use level of 0.9% AHTN (this level is seen only in toilet blocks – the highest level in all other household products is 0.12%) the maximum oral exposure resulting from ingestion would be 90 mg HHCB. Studies of the acute oral toxicity demonstrate that the toxic dose of HHCB is many times higher than this, even for a small child.

Eye and dermal contact are not considered significant because HHCB is neither an eye nor a skin irritant and the small amount of HHCB in any given product would not contribute significantly to the irritancy of the product as a whole.

5.1.3.11 Overall exposure

In the unlikely event of maximum exposures from all of the above sources excluding possible intake from fish and drinking water, the total exposure to HHCB from its use in household cleaning products would be 0.07 µg/kg bw/day. The estimated intake from drinking water is negligible compared to other exposures and the estimated indirect exposure from fish is 1.4 µg/kg bw/day.

5.1.3.12 Special consideration of exposure to nursing infants

Because HHCB has been found in human milk samples, consideration of possible risk to the nursing infant from the resulting exposure should be considered even though there is no evidence that the occurrence in the milk is the result of the use of household cleaning products. Indeed, in a study of over 100 nursing mothers, there was no statistically significant correlation of the occurrence or levels of HHCB with the use of household products.

In this study (Sönnichsen, et al. 1999 - See section *5.2.1.9.* Additional data - Human milk studies), a mean level of 80 µg HHCB/kg milk fat and a maximum level of 1316 µg/kg and a mean fat level of 3.67% was found. The exposure to babies is calculated according to the WHO (1998) and is described here. For the first three months in life, an infant consumes an average of 120 g/kg bw/day. After three months of age, the volume consumed per unit weight of the infant decreases with increasing age. By multiplying the concentration (given as mg/kg or mg/l) of a particular substance in whole milk by a factor of 0.12, the approximate daily intake of the substance in mg/kg bw/day can be estimated. Using the mean fat content was 3.67 % it is possible to calculate the uptake of HHCB via mother's milk ass follows:

Mean: 80 µg HHCB/kg milk fat = 80 x 0.120 x 0.0367 = 0.35 µg HHCB/kg bw/day.

Maximum: 1316 µg HHCB/kg milk fat = 1316 x 0.120 x 0.0367 = 5.8 µg HHCB/kg bw/day.

5.2 Hazard Assessment

5.2.1 Summary of the available toxicological data

5.2.1.1 Acute Toxicity

a) Acute Oral Toxicity

Galaxolide 50 (Non-GLP; 65% HHCB) in diethyl phthalate (DEP)) was administered undiluted (hence, there was variation in volume of dosing) by oral intubation at doses of 0.215, 0.464, 1.0, 2.15 or 4.64 g/kg bw (equivalent to doses of HHCB of 0.14, 0.30, 0.65, 1.4, 3.0 g/kg when corrected for the 65% dilution) to groups of 5 female Charles River Sprague Dawley rats (initial bodyweight 104–141 grams) that were then observed for mortality and signs of effects for 7 days. There was one death as a result of gavage error at 1.0 g/kg but no deaths at any other dose. One animal at 2.15 g/kg appeared distressed shortly after dosing but appeared normal after 2 hr. There were no effects at the high dose. An LD_{50} of >4.64 g/kg bw (equivalent to >3 g/kg bw HHCB) was calculated (Minner and Foster, 1977). This study was conducted prior to GLP and OECD guidelines but was conducted according to acceptable procedures at the time.

In a limitedly reported oral gavage non-GLP study, Galaxolide 50 (65% HHCB in DEP) was administered to 10 rats at a dose of 5000 mg/kg bw (actual dose of HHCB – 3250 mg/kg bw) followed by a 14 day observation. At the end of a 14-day observation period, only one rat had died (on day 2). The oral LD50 can be listed as >3250 mg/kg bw (Moreno, 1975). This study was conducted prior to GLP and OECD guidelines and specific details regarding the study are not available. However, the study was reported by the Research Institute for Fragrance Materials (RIFM) and was conducted by a standard protocol that was state of the art at the time (Personal communication, RIFM).

b) Acute Inhalation Toxicity

There are no test data available to evaluate the acute inhalation toxicity of HHCB. Two 90-day inhalation studies with rats of compounded fragrance oils containing HHCB at levels resulting in the inhalation of 5.7 $\mu g/m^3$ and 132 $\mu g/m^3$ showed no adverse effects (see below). Because of the low volatility and low use levels of HHCB, inhalation is not considered a significant exposure pathway compared to the dermal exposure.

c) Acute Dermal Toxicity

Galaxolide 50 (65% HHCB in DEP) was administered undiluted by inunction to the shaved skin (area not reported) of groups of five female Charles River Sprague Dawley rats (initial bodyweight 108–187 g) in doses of 0.464, 1.0, 2.15, 4.64 or 10.0 g/kg bw (equivalent to 0.30, 0.65, 1.4, 3.0, 6.5 g/kg HHCB) that were then observed for 7 days. There were no deaths at any dose but all animals in the high dose group exhibited urine staining on their fur. A dermal LD_{50} of >10.0 g/kg bw (equivalent to >6.5 g/kg bw HHCB) was reported (Minner and Foster, 1977). This study was conducted prior to GLP and OECD guidelines but was conducted according to acceptable procedures at the time.

In a limitedly reported dermal acute toxicity study, Galaxolide 50 (65% HHCB in DEP) was applied to the skin of groups of 7 albino rabbits at a dose of 5000 mg/kg bw (equivalent to 3250 mg/kg bw HHCB). There were no deaths at that dose therefore the dermal LD50 can be listed as >3250 mg/kg bw. In all animals, moderate redness of the skin was seen, in 6/7 animals moderate oedema of the skin and in 1/7 animals slight oedema of the skin was seen.

No control animals with only solvent were included. (Moreno, 1975). This study was conducted prior to GLP and OECD guidelines and specific details regarding the study are not available. However, the study was reported by the Research Institute for Fragrance Materials (RIFM) and was conducted in accordance with OECD Guideline 401. No solvent would have been used since the material as tested was a liquid (Personal communication, RIFM).

d) Acute Toxicity – other routes

Groups of five female rats (Charles River Sprague Dawley – weighing 106-155 g) were dosed with Galaxolide 50 (65% HHCB in DEP) by intraperitoneal injection at doses of 0.1, 0.215, 1.0 or 4.64 g/kg bw, (equivalent to 0.065, 0.14, 0.65 or 3.0 g/kg bw HHCB) and observed for 7 days. At 1.0 g/kg, 3 animals were observed to be in a depressed condition within two hr of dosing but returned to normal at 24 hr. There were no deaths at this dose. Lethargy and depression were observed in 4/5 animals at the high dose within 2 hr and all were found dead at 24 hr. The remaining animal was prostate at 24 hr and found dead the next day. Based on these observations an IP LD_{50} of 3.16 g/kg bw (equivalent to 2.1 g/kg bw HHCB) was calculated (Minner and Foster, 1977). This study was conducted prior to GLP and OECD guidelines but was conducted according to acceptable procedure at the time.

In a range finding study in preparation for a micronucleus test (see section 4.1.2.7 below), groups of 5 male and 5 female ICR mice were dosed with 500, 1000, 3000 or 5000 mg/kg bw of HHCB in corn oil by intraperitoneal injection at a constant volume of 20 ml/kg bw. Based on no mortality at 500 or 1000 mg/kg bw and deaths of 4/5 males and 5/5 females dosed at 3000 mg/kg and all 5 male and female mice dosed at 5000 mg/kg bw died, a LD_{50} of 2135 mg/kg bw was calculated by probit analysis (Api and San, 1999, Gudi and Ritter, 1997).

Conclusion

HHCB is considered to have a low order of toxicity based on oral and dermal acute studies in the rat showing less than 50% mortality at the highest dose administered (3250 mg/kg bw orally and 6500 mg/kg dermally.

5.2.1.2 Irritation

a) Skin irritation

A series of GLP compliant studies, have been performed according to directive 79/831EEC using New Zealand White female rabbits.

In the first of these studies, 0.5 ml of Galaxolide (65% HHCB in DEP) was applied over an area of approximately six cm^2 for 4 hr under semi-occlusive lint patches (held in place with Elastoplast plastic adhesive bandage 10 cm wide) on the dorsal skin (clipped free of fur) of three rabbits for a period of 4 hrs. Undiluted DEP and benzyl benzoate (BB) were similarly applied to groups of three rabbits. Scores for erythema and oedema per animal were given after 1, 24, 48, 72 and 168 hrs and the average scores per animal for erythema and oedema over 24, 48 and 72 hrs were calculated. The results for Galaxolide were an erythema score of 1.3 (on all three) and an oedema score of 0.4 (highest score 1). After 168 hrs, slight desquamation of the skin surface at the treated site was seen in all three rabbits. For DEP and for BB, the scores were zero for erythema and oedema on all three rabbits. (Haynes, 1984).

In another study 0.5 ml of Galaxolide 50 (65% HHCB in DEP), DEP and BB was placed evenly over a 2.5 cm^2 of surgical lint, which was then placed on the skin of each of 4 rabbits and held by an Elastoplast adhesive bandage of 10 cm wide for a period of 4 hrs. Scores of

skin results were given after 1, 24, 48, 72, and 168 hrs. An average score (average of all 4 animals over 24, 48, and 72 hrs) for erythema of 2.1 and for oedema of 1.5 was calculated for Galaxolide. The average scores for DEP were 0.2 for erythema and zero for oedema while the corresponding scores for BB were 1.2 and 0.4. (Haynes, 1985)

In the final study, 0.5 ml of either undiluted or 50% solutions of Galaxolide 50 DEP (65% HHCB in DEP) or Galaxolide 50 BB (65% HHCB in benzyl benzoate) were placed evenly over a 2.5 cm^2 patches of surgical lint. These patches were then placed on the skin of 4 rabbits and held by an adhesive bandage of 10 cm wide for a period of 4 hrs. The average erythema/oedema scores calculated over 24, 48 and 72 hours were 1.8/1.3 for 100% Galaxolide 50 DEP, 1.3/0.3 for 50% Galaxolide 50 DEP in DEP, 1.8/0.8 for 100% Galaxolide 50 BB, and 1.3/0.7 for 50% Galaxolide 50 BB in BB, respectively. In this study, there were no solvent controls. However the results can be compared to the results with the two solvents seen in the previous two studies (Haynes, 1986).

Non-GLP tests for irritancy were conducted with undiluted Galaxolide-50 (65% HHCB in DEP) or a 50% solution of Galaxolide-50 in SDA39 alcohol (final HHCB concentration was 32.5%) on 3 albino rabbits (strain not specified). A single application of 0.5 ml of the test material was applied to the skin (area specified as 2x2 with no units), which had been clipped free of hair and, on a site that was abraded so as to penetrate the stratum corneum and an unabraded site. The site of application was covered with a Webril patch and sealed with Blenderm Surgical tape for 24 or 72 hr during which the rabbits were immobilized in racks. At the end of the 24-hr patch period and again 48 hr later, the sites were scored according to Draize. Average scores of 1 for erythema were observed at both 24 and 72 hr and at both abraded and unabraded sites but no oedema was seen with the 50% solution but no erythema or oedema was seen with Galaxolide 50. It was concluded that the 50% solution of Galaxolide 50 was a moderate irritant but the 100% was not a primary irritant under the conditions of this test (Levenstein, 1973).

In another non-GLP test under identical conditions to the preceding, three rabbits were treated with solutions of 25%, 50% Galaxolide 50 (equivalent to 16, 33 or 65% HHCB) in Alcohol SDA 39C as well as undiluted Galaxolide 50 (65% HHCB in DEP) for 24 or 72 hr. No solvent control was reported. The 25% solution produced no erythema or oedema at either abraded or unabraded sites. The 50% solution produced a score of 1 for erythema at 24 hr on abraded skin but no erythema or oedema at any other site or time period. It was concluded that this solution could be considered a very mild irritant. The undiluted Galaxolide 50 produced scores of one for erythema at 24 hr on both abraded and unabraded sited but no erythema at 72 hr and no oedema at any time on either site. It was concluded that undiluted Galaxolide 50 could be considered a mild irritant under these test conditions (Levenstein, 1975).

During the induction phase of Human Repeated Insult Patch Test (HRIPT) for sensitisation, a semi-occlusive patch of 100% neat HHCB was applied on the upper arms of the 42 subjects for 24 hr three times per week for three weeks. 0.5 ml of the test substance was applied to a 1x1 inch Webril patch, which was affixed to the centre of a 1x2 inch elastic bandage and applied to the upper arms of the panelists. Reactions were scored at 24 and 72 hr after patch removal. No irritation was observed in any of the 42 subjects (group 117) even after repeated occlusive applications of undiluted material (Guillaume et al., 1973b).

Forty subjects were tested with HHCB and evaluated for irritation as part of sensitisation study. Nine semi-occlusive induction applications of 3.75% Galaxolide were made on the

upper arms of the subjects, 3 times a week for 3 weeks. Little or no primary irritation was observed under the conditions of this study (Rubenkoenig and Ede, 1964).

b) Eye irritation

Galaxolide (65% HHCB in DEP) was tested in the eyes of 6 rabbits by a procedure essentially equivalent to OECD 405 (no observation at 1 hr and no wash at 24 hr). 0.1 ml of Galaxolide was instilled into the right eye (the left serving as control) of healthy young adult albino New Zealand rabbits. Both eyes were scored according to the method of Draize at 24, 48, 72, 96 and 168 hr. Four rabbits had no ocular changes at any time. One had a small central opacity (score 2) at 24 hr, which was fainter at 48 hr and cleared at 72 hr. The same animal showed also score 1 for effect on the iris at 24 hr only and slight conjunctival redness and discharge (both score 1) at 24 hr only. Another animal had slight redness of the conjunctivae (score 1) at 24 and 48 hr but no effects at later times. Primary eye irritation score at 24, 48, and 72 hr was 3.5, 1.17 and 0 (average of three time points was 1.6). Based on the irritation scores the test material (Galaxolide) is considered as practically nonirritating to the eye. (Sauer, et al, 1980).

In an incompletely reported study, 0.1 ml of Galaxolide 50 (65% in DEP) was tested in the eyes of three rabbits with an observation time of 168 hours by a method that seemed similar to that above. No irritation was seen in all three rabbits at any observation time (Levenstein, et al., 1975).

In an incompletely reported study, 0.1 ml of a 50% solution of Galaxolide 50 (65% HHCB in DEP) in ethanol (final concentration of HHCB was 32.5%) was tested in the eyes of three rabbits with an observation time up to 168 hr by a method, which seemed to be similar to those above. In all three rabbits conjunctival irritation (redness, chemosis and discharge) (scores 2 to 1 at 24 hr) was seen which was cleared in two rabbits by 48 hr and in the third by 168 hr. Primary eye irritation index for one rabbit was 4.7 (average 24, 48, and 72 hr) and for the other two 2.7 and 2.7. The rabbit with the highest irritation index in this study started to show corneal opacity (score 2) with an area score of 2 (>25%<50%) after 96 hr persisting up to termination of the study after 168 hr. In the control study with ethanol, the primary eye irritation index was 7.3, 5.3 and 2.3 over 24, 48, and 72 hrs (Levenstein et al, 1973).

In a similar study, a dilution of Galaxolide (65% HHCB in DEP) in ethanol (concentration unknown) (Wolven and Levenstein, 1963) was tested with an observation time up to 168 hr. Conjunctival irritation (redness, chemosis, discharge) with scores 1 to 2 was seen in all three rabbits at 24 hr. Discharge disappeared after 48 hr, whereas redness and chemosis persisted up to 96 hr.

As ethanol has eye irritant potential, the relevance of these last two studies is questionable.

Conclusion

HHCB is not irritating to the skin or eyes up to and including 100% concentration.

5.2.1.3 Phototoxicity

Because HHCB absorbs in the UV region, several studies to detect a possible photoirritation hazard have been conducted (see Table 2). Several of these were designed for method development. Up to now, there are no validated in vivo tests for phototoxicity. However, draft testing guidelines for photoirritation have been circulated by OECD both for in vivo as well as in vitro tests. These draft guidelines have been used to facilitate the interpretation of the studies cited below. The test described in the draft guideline for in vitro testing has also been discussed and adopted by the EU-SCCNFP. Also the (USA) Cosmetic, Toiletry, and

Fragrance Association (CTFA) has developed a guideline for photoirritation studies. One test has been performed according to this CTFA guideline.

Studies in vivo

Table 2. In vivo animal studies of the phototoxicity of HHCB

Species	GLP	Results	Reference
Rabbits and guinea pigs	No	Slightly positive reactions at concentrations of 13 or 32%	Sato et al., 1978
Guinea pigs	No	Positive in 5/20 animals at 6.5%	Guillot, 1985
Mice	No	Negative at 65%	Forbes, et al, 1978
Rabbits and guinea pigs	No	Positive reactions observed at >3.25%	Ogoshi, et al, 1980, 1981

In tests for photoirritation, 0.02 ml HHCB (purity unknown) in ethanol or diethyl phthalate (DEP) were applied evenly to 1.5 x 1.5cm areas on both sides of the shaved, depilated backs of rabbits or guinea pigs. One side of the animal's back was used as a control side and covered with aluminum foil. Three rabbits and 3 guinea pigs (strains not reported) were treated with 5 (in ethanol), 10, 20 and 50 % (in DEP) of a commercially available sample of HHCB (equivalent to 3.25% in ethanol and 6.5, 13 or 32.5% HHCB in DEP). The rabbits were treated only with the lower 2 concentrations. Treatment was followed (time after dosing not specified) by glass-filtered UV-A irradiation for 110 min from six Toshiba 40 WFL BLB lamps (300-400 nm; peak at 360 nm) at a distance of 10 cm.. Readings were taken at 24, 48 and 72 hr after irradiation. The difference between the average scores of irradiated and non-irradiated sites was evaluated to determine phototoxicity. No significant effects were observed at either of the 2 lower doses of HHCB with rabbits or guinea pigs. The 2 higher doses produced reactions only in guinea pigs. At 32.5% in DEP the average score over 24, 48 and 72 hrs for erythema/oedema was 2.2/1.7 compared to 0.5/0.0 at control sites (presumably solvent only) At 13% in DEP the average score for erythema/oedema was 0.8/0.6 compared to no reaction at control site. No positive control was tested. According to classification into categories given by the author of the study, based on the scores, the 32.5% solution of HHCB is concluded to be moderately phototoxic and the 13.5% solution to be very weakly phototoxic. (Sato et al., 1978).

The phototoxic potential of HHCB (purity unknown) was evaluated in 10 male and 10 female young adult albino Dunkin-Hartley guinea pigs. The animal's fur on back and flank was clipped and depilated, 24 hr prior to dosing. A single application of a 10% solution of a commercial sample of HHCB (65% in DEP) (so the actual concentration was 6.5%) in ethanol (0.5 ml) on a gauze pad of 2 cm^2 was applied to the skin on the back for 1.3 hr. The gauze pad was kept in contact with the skin by an adhesive hypoallergenic patch under an occlusive aluminum foil sheet of 5x5 cm. Another (not-treated) part of the dorsal skin was also covered with aluminum foil to protect it from unwanted irradiation. Three male and two female guinea pigs were maintained as a control group and were treated with the same solution of HHCB but were not irradiated. The treated patches were irradiated for 5 min using a system of 2 fluorescent lamps with continuous UV-A spectral emission of 310-400 nm (peak at 360 nm) and 285-350 nm (UV-B; peak at 310 nm) delivering energy of 12.5 J/cm^2 (99% UV-A; 1% UV-B), as dosimetrically determined. This amount of irradiation was

the minimal erythematous dose. Readings of erythema and oedema were carried out 6 and 24 hr after irradiation. The readings were performed in a blinded way. Erythema and oedema were scored on a 5-unit scale (no effect – very pronounced effect). Erythema and oedema scores were considered positive if it was 2 units greater than the one attributed to the control sites. Negative and doubtful scores were equal to that or slightly higher than that in the control group, respectively. Positive histopathological readings were those representing "sun burned" type of lesions. The final score for each treated and irradiated animal was made based on both macroscopic and histopathological examinations. Macroscopically, in 2/20 and in 12/20 animals, respectively, a positive or a doubtful response was observed. Histopathology confirmed that 5 of these responses were positive and 15 were negative. In conclusion, evidence of phototoxicity was observed in 25% of the animals receiving a dose of 6.5% HHCB (Guillot et al., 1985).

An aliquot of 20 µl of Galaxolide (65 % HHCB in DEP) was applied to 5 cm^2 of normal skin in SKF Hairless-1 mouse (number of animals not given). At 30 min after the application, the centre 1 cm diameter circle of the application site was irradiated for 30 min with simulated sunlight using a filtered (Schott WG 320) Osram XBF 6000 w Xenon lamp, or was irradiated for 1 hr with a bank of F40T12BL fluorescent black light, (glass-filtered to eliminate "sunburning UV light" (<320 nm)). A sample of citrus lime oil was used as a positive control. Skin reactions were assessed at 2, 4, 24, 48 and 72 hr after irradiation. No skin phototoxic reactions were observed with HHCB but the positive control gave symptoms of phototoxicity at 24-72 hr post irradiation exposure (Forbes, et al., 1978).

(The following was taken from a symposium paper presented in 1980 and from the published paper, in Japanese, presumably reflecting the same data.) Galaxolide 50 BB (65% HHCB in benzyl benzoate) at a concentration of 1, 5, 10 or 20% in either petrolatum or 99.5 % ethanol (actual HHCB concentrations 0.65 %, 3.25 %, 6.5 % and 13 %) was tested in rabbit and guinea pig. The number of animals used in the study is not included in the study summary. 50 µg sample of the test mixtures were applied to 4 cm^2 shaved skin area for 2 hr and then the animals were irradiated for 30, 60, or 120 min by five Toshiba FL20sBLB fluorescent lamps, 300-430 nm. Non-irradiated sample patches were used as control sites. The total irradiation dose was 1.6 -7.6 J / cm^2. Skin reactions were assessed 3 days after irradiation. There were no phototoxic effects observed at 0.65 % and 3.25%. Phototoxicity was observed at 6.5% and 13% only in guinea pigs after 120 min of irradiation. In rabbits, 6.5% produced phototoxic reactions after 60 min of irradiation and 13 % produced phototoxic reactions after 30 min of irradiation. No data were given on strength of the reactions. Irradiated test materials had a stronger irritating effect than the corresponding unirradiated test material. Therefore, the strength of the reactions tended to depend on the quantity of the irradiated light and the concentration of the substance tested. Based on these data, Galaxolide 50 BB was classified by the authors as equivocal (±) as to phototoxicity at the highest concentration of 20% (equivalent to 13% HHCB) (Ogoshi, et al., 1980, 1981).

Human

Table 3 In vivo human studies of the phototoxicity of HHCB

Number of subjects	GLP	Results	Reference
26, 25 & 9	No	Negative at 65%	Lindstrum et al.,, 1978a, 1978b, 1978c
10	No	Negative at 6.5%	Harrison and Stolman, 1986
10	No	Negative at 6.5%	Gabriel and Mark, 1987
26	No	Negative at 6.5%	Folk and Dammers, 1987

Number of subjects	GLP	Results	Reference
10	No	Negative at 6.5%	Shanahan and Alworth, 1987

There is no standardized protocol for conducting phototoxicity screening in humans. Because of this, several tests were conducted in different laboratories using their standard protocol and standard operating procedures.

Galaxolide 50 (65% HHCB in DEP) undiluted or as a 50 or 25% solution in ethanol was applied under occlusive patches of approximately 2 cm^2 area on the backs of 26 female volunteers. The volume of the material applied for each patch ranged from 0.2 to 0.4 ml. Patches were allowed to stand for 30 min for the evaporation of the solvent-ethanol before application. Control exposures to natural sunlight were conducted at the approximate minimal erythematous dose (MED) for each subject. No solvent controls were included. The MED for an individual subject is defined as the exposure time in min that induced a faintly perceptible erythema at the irradiated site, 16 to 20 hr following sun exposure. After *ca*. 21 to 23 hr of HHCB treatment, the treated skin sites were scored, and treated sites as well as untreated sites were exposed to natural sunlight at the MED. 16 and 40 hr after exposure to sunlight the HHCB treated and control sites were evaluated. A positive phototoxic reaction is characterised by sharply demarcated erythema at a test site, which has been treated with test material and 1 MED of sunlight. Subjects who did not show perceptible erythema at the untreated site were excluded from the study. No evidence of phototoxicity was observed after skin exposure to HHCB containing samples followed by irradiation with natural sunlight. Positive control sites exposed to a solution of 8- methoxypsoralen in ethanol showed signs of phototoxicity (Lindstrum et al., 1978a).

Another study following the same method was conducted in 25 female panelists using 0.3 ml per site of Galaxolide 50 BB (65% HHCB in benzyl benzoate) undiluted showed no evidence of phototoxicity (Lindstrum et al., 1978b).

In a third study following the same method with 9 female panelists, 0.4 ml per site of undiluted HHCB, , Galaxolide 50 IPM (65% HHCB in isopropyl myristate), or Galaxolide BB (65% HHCB in benzyl benzoate) were tested using the same natural sunlight method, with no evidence of any phototoxicity (Lindstrum et al., 1978c)

The phototoxic potential of Galaxolide 50 (65% HHCB in DEP) was evaluated in 10 volunteers (2 males and 8 females) who were treated with duplicate occlusive patches (Redi-Bandage) containing 0.2 ml of a 10% solution of Galaxolide 50 in ethanol/DEP (3:1) (resulting concentration of HHCB - 6.5 %) applied to each volar forearm (area not reported) for 24 hr. After patch removal, one of the forearms was irradiated with UVA from 4 F40BL fluorescent tubes (output at 360 nm of 1.23 W/10 nm of wavelength). The dose delivered was 0.22 J/cm²/min. The sites were scored immediately after irradiation and at 24 and 48 hr later. No visible reactions were seen at any time on any subject (Harrison and Stolman, 1986).

Galaxolide 50 BB (65% HHCB in benzyl benzoate) and Galaxolide 50 DEP (65 % HHCB in DEP) were tested as a 10 % solution in ethanol/DEP (3:1) (actual concentration of HHCB - 6.5%). The vehicle control was 75% ethanol: 25% DEP. Before application of the preparation for each of the subjects the Minimal Erythemal Dose (MED) was determined using UV light irradiation from a xenon arc solar light simulator. Subsequently, areas on the back of 10 female subjects (ages 18-39) were stripped 3 times to remove the superficial stratum corneum. This was followed by application of 20 µl of the test solution to 2 designated test sites, each approximately 1.5 cm in diameter. In addition, three of the 10 subjects were randomly

selected and treated with 20 µl of a 0.2 mg/ml solution of 8-methoxypsoralen in ethanol as a positive control. After 30 min of exposure to the test material, one site was irradiated with UVA followed by UVA + UVB calibrated xenon lamp and the other site remained unirradiated. The test material was maintained on the skin sites. A third site was used as untreated, irradiated control site (UVA + UVB only). Vehicle treated irradiated and unirradiated control sites were included. Based on the previously determined MED, each individual subject was exposed to UVA light for a time period of 10 MED equivalents. Followed by exposure, to 0.5 MED of UVA ± UVB light. The sites were scored 5 min after irradiation and thereafter were lightly covered. The sites were reexamined at 3, 24, 48 and 72 hr after irradiation. Sites were re-covered after the 3 hr reading through the 24 hr reading and were uncovered thereafter. There were no significant reactions to either preparation of HHCB but all three subjects exposed to 8-MOP showed clear positive reactions (Gabriel and Mark, 1987).

In another phototoxicity study, 26 volunteers (male and female Caucasians) received single applications of duplicate sets of patches with 0.3 ml of a 10% ethanol/DEP (3:1) solution of Galaxolide BB (65% HHCB in benzyl benzoate), (final concentration of HHCB of 6.5 %). The test material was applied to areas of approximately 1.5 cm in diameter (~1.75 cm^2) on the back under occlusion for 24 hr. The test site was irradiated with 16-20 J/cm^2 of UVA from a filtered xenon arc solar simulator within 10 min after patch removal. Prior to irradiation, any excess test material remaining on the skin was wiped off with a wet towel. All sites were evaluated 1, 24, 48, and 72 hr after irradiation. Treated unirradiated control sites, and vehicle treated irradiated and unirradiated control sites were included. No significant reactions due to HHCB were observed (Folk and Dammers, 1987).

According to another phototoxicity study, a 10% solution Galaxolide 50 BB (65% HHCB in benzyl benzoate) in ethanol/DEP (3:1), (resulting concentration of HHCB - 6.5%), was tested in ten volunteers (8 females and 2 males). Inner aspects of their forearm were tape stripped 3-4 times to remove the outer stratum corneum. 0.2 ml of the test material was placed directly on the skin (surface of treated skin area was not reported), which was then subjected to ultraviolet radiation, receiving a UVA light dosage over a period (~60 min) sufficient to deliver 15-20 joules of energy. The light exposure was from fluorescent bulbs. The contact site was then covered with an occlusive patch (Parke-Davis Readibandage^R), with ~0.2 ml additional test material for 24 hr. 0.2 ml of the test material on a Parke-Davis Readibandage^R occlusive patch was applied to non-irradiated control sites and left in place for 24 hr. No significant reactions to HHCB were observed (Shanahan and Alworth, 1987).

In vitro

Table 4. In vitro studies of phototoxicity of HHCB

Study Type	GLP	Results	Reference
Mouse Fibroblasts 3T3 Assay	Yes	Negative	Harbell, et al. 2001
Photohemolysis of human RBCs	No	Positive	Sugiyama, et al 1994
Yeast	No	Positive	Sugiyama, et al. 1994
Yeast	No	Negative	Tenenbaum, et al. 1984
Yeast	No	Positive	Forbes, et al, 1978
Yeast	No	Negative	Weinberg and Springer, 1981

Study Type	GLP	Results	Reference
Yeast	No	Positive	Bagley, et al 1988

In a GLP compliant study according to the draft OECD guideline / adopted EU-SSCNFP guideline mentioned above, Balb/c 3T3 mouse fibroblasts were exposed to 50 µl aliquots of HHCB (purity not reported) in Hank's Balanced Salt Solution (HBSS) containing 0.5% ethanol (concentrations of 1.77 – 100 µg/ml) for 1 hr followed by irradiation with UVA light for 50 minutes for a total irradiation dose of 5 J/cm^2. Duplicate slides were kept in the dark for the 50-minute period. After the irradiation period, the test solutions were decanted from the plates and the cells were washed with HBSS. Assay medium was then added to the cells and the cells were incubated for 24 hr at which time the assay medium was decanted from the cells and 100 µl of filtered Neutral Red solution added. After a 3 hr incubation, the cells were washed, scored for Neutral Red uptake and the IC_{50}, the Mean Photo Effect (MPE) and Photo-Irritation Factor (PIF) were calculated. The average (over 2 runs) IC_{50} for HHCB was 8.84 µg/ml with and 11.2 without irradiation. The MPE was 0.016 (<0.1 is considered non-phototoxic) for each run and the PIF was 1.32 and 1.12 (<2.0 is considered non-phototoxic). Chlorpromazine was tested as a positive control. The average IC_{50} was 30.1 µg/ml with irradiation and 1.84 without. The MPE was 0.64 for each run and the PIF was 18.36 and 13.62 (Harbell, et al. 2001).

In vitro photohemolysis studies with Galaxolide (65% HHCB in DEP) and red blood cells were conducted. Cells obtained from healthy volunteers were washed, centrifuged, and suspended in a buffer. HHCB in serial five fold dilutions was added to a microplate along with the red blood cell suspension and irradiated with long wavelength ultraviolet light (UVA, 25 J/cm² - time not given). Hemolysis of the red blood cells was measured after irradiation. HHCB produced positive effects as indicated by 15.9 % photohemolysis of the red blood cells. Details on the concentration of HHCB used are not provided. None of the three (*in vivo*) positive controls, 8-methoxypsoralen (8-MOP), 5-methoxypsoralen (5-MOP) and 6-methylcoumarin (6-MC) gave any measurable photohemolysis nor was classified as negative in this assay (Sugiyama, et al., 1994).

The same workers conducted an *in vitro* yeast (*Saccharomyces cervisiae*) growth inhibition assay for photoirritation with a five-fold dilution of Galaxolide (65% HHCB in DEP). The plates were incubated for 72 hr after irradiation with 50 J/cm^2 of UVA (time of irradiation not reported). A 2.1 mm growth inhibition was observed on the plates indicating positive effects (a positive reaction was defined as a 2 mm inhibition) were seen with HHCB. Details on the concentration of HHCB used and applied in serial five-fold dilutions are not provided. Three positive controls, 8-MOP, 5-MOP and 6-MC gave inhibitions of 14.1, 10.0 and 6.4 mm, respectively (Sugiyama, et al., 1994).

In a similar photoirritation assay, *Saccharomyces cervisiae* cultures were covered with filter paper impregnated with a 5% solution of Galaxolide (65% HHCB in DEP - 3.25% actual concentration of HHCB) in methanol. The plates were exposed to UVA radiation for 18 hr (320-400 nm; peak 350 nm) and incubated for 48 hr. 8-MOP (0.0005-0.001%) was used as a positive control. No effects were seen with HHCB but 8-MOP gave positive results (Tenenbaum, et al., 1984).

Galaxolide 50 (65% HHCB in DEP) was evaluated in an *in vitro* test using plates seeded with yeast (*Saccharomyces cervisiae*). The Galaxolide 50 was applied to filter paper plates and

placed on culture media, which had been seeded with yeast. The plates were then grown for 24 hr under 10 W/m² UVA and observed at 24-hr intervals for four days. A 6.5% v/v/ Galaxolide solution after irradiation caused growth inhibition in the area adjacent to the plate and was concluded to be positive in the yeast assay. This was a marginal positive value. Inhibition greater than 2 mm was considered a positive response. HHCB resulted in a growth inhibition of 2.1 mm. Details on the concentration of HHCB and the solvent used to dissolve HHCB are not provided (Forbes, et al, 1978).

An *in vitro* study on Fleischman's active dry yeast (*Saccharomyces*) was conducted with 0.1, 1.0 and 10% solutions of Galaxolide (65% HHCB in DEP) in methanol. The solution (40 µl) was added uniformly to a paper disk and allowed to dry for 15 min. The disk was then applied to the yeast suspension, and the plate was then exposed to UVA (320 - 400 nm, peak 370 nm) for 18 hr with a flux of 1.5 W/cm². Forty-eight hr after inoculation or when contrast is adequate, zones of inhibition were measured. The positive control was 8-MOP but several other known phototoxicants gave positive reactions as well. No effects were observed up to the maximum tested concentration of 10% Galaxolide (equivalent to 6.5% HHCB) in methanol (Weinberg and Springer, 1981).

A modified *in vitro* yeast cell assay was conducted using *Saccharomyces cervisiae*. Paper discs were treated with 25 µl of a solution of Galaxolide (65% in DEP) (lowest concentration tested - 0.3%; higher concentrations not given) in methanol and then placed on plates that had been seeded with yeast. The plates were then grown for 18 hr under UVA (320 - 400 nm, peak at 340 - 360 nm) and then incubated at 32° C. After incubation, zones of inhibition were measured. 8-MOP (concentrations of 0.001% to 0.01%) was the positive control. Positive effects due to Galaxolide were observed at the lowest dose tested (no details of the effect at this or higher doses were given) (Bagley, et al., 1988).

Conclusion

No phototoxic effects were observed in humans in several different tests with concentrations up to 65% regardless of the experimental conditions applied.

HHCB was positive in 4 out of 7 of the *in vitro* phototoxicity tests however in the only one of which conformed to presently accepted methodology, an *in vitro* study according to the methodology of EC/COLIPA, gave a negative result. There is evidence of weak phototoxicity in guinea pigs and rabbits with an apparent no effect level of at least 3.25%, however, in the more accepted test on hairless mice, a commercial preparation (~65%) gave no effects.

Based on all data available HHCB is not considered to be photoirritant as used in household products.

5.2.1.4 Sensitisation including photosensitisation

a) Sensitisation

Studies in animals

Galaxolide (65% HHCB in DEP) has been subjected to a non-GLP guinea pig maximization test. The used doses of Galaxolide were 0.5% in 0.01% dodecylbenzene sulphonate in 0.9% saline (DOBS/saline) for the intradermal injection, 100% for the induction patch, and 25% in 70% acetone/30% polyethylene glycol 400 (acetone/PEG400) for the challenge patch. These doses were selected based on preliminary irritation tests using 0.1, 0.25, 0.5, 1.0 and 2.0% Galaxolide concentrations for intradermal injections, however the selection criteria were not

clear. The actual concentrations of HHCB are 0.325%, 6.5%, and 16.25%, respectively. Ten (six male, four female) Albino Dunkin/Hartley guinea pigs (weight 316-350g) were tested on a 2cm x 4cm area of skin in the dorsal shoulder area, clipped free of fur. Induction consisted of a 0.1 ml intradermal injection of 0.325% HHCB in DOBS/saline and 0.1 ml 50% Freund's Complete Adjuvant in 0.9% saline. This was followed one week later by a 48 hr occluded patch (filter paper attached by adhesive tape to polythene backing) saturated with 65% HHCB The patch was applied at the same 2 cm by 4cm area after freshly shaving the skin. Challenge applications were made 14 days later at a freshly shaved naïve site by saturation of an 8mm diameter filter paper patch with 16.25% HHCB in 70% acetone/30% PEG 400. Eight animals were treated as controls and received induction and challenge treatments similar to the test pigs minus the test material. Two repeat challenges at weekly intervals were conducted. At 24 hours, very faint erythema (score 0.5) was found in 2/10 animals at challenge 1, 3/10 animals at challenge 2, and 1/10 at challenge 3. At 48 hours, 3/10, 1/10 and 0/10 had very faint erythema. At challenge at 24 hours, only one animal showed very faint erythema to faint erythema. Except for one equivocal response in one animal, no evidence that the material was a sensitiser was seen (Basketter, 1996).

In a GLP compliant study, Galaxolide (65% HHCB in DEP) was tested for its allergenic and photoallergenic potential in 12 albino Hartley strain guinea pigs (418 to 487 g) with Freund's adjuvant injection at the shaved interscapular region. Four injections of 0.1 ml Freund's Complete Adjuvant were administered to the four corners of a 9 cm^2 shaved site. 0.1 ml of 1% Galaxolide in ethanol (resulting in an actual HHCB concentration of 0.65%) was dermally applied to the site. The used doses were selected based on preliminary photoirritation studies to be the maximum non-photoirritant or slightly photoirritant doses. After 25 min, the sites were exposed to ultraviolet light using fluorescent black lamps for about 1 hr and 34 min (ca. 1.8 mW/cm^2; 10 J/cm^2). The procedure, excluding adjuvant injections, was repeated 24 hr later. A control group of 12 guinea pigs (422 to 547 g) were treated in the same way except that the treatment with test substance was replaced by treatment with solvent. Ten to 14 days after induction, the guinea pigs (both test and control animals) were challenged with 1, 0.3 or 0.1% Galaxolide in ethanol (actual HHCB concentrations 0.65%, 0.2%, and 0.065%) by dermal application to the shaved lumbar region. Thirty min later, the animals were irradiated as above, after which the test material was applied to fresh sites to check for contact sensitivity, and the sites scored at 24 and 48 hr. A second challenge was carried out 6 or 7 days later. In 1/12 a very faint trace of erythema was found at 1.0% and 0.3% Galaxolide with UVA at challenge 1 and 2. Under the conditions of this test, Galaxolide is not a photosensitiser in guinea pigs (Parish, 1988). This study was conducted prior to but essentially similar to the OECD 406 guideline except only 12 animals were used and another scaling of grades was used.

Studies in humans

A Human Repeated Insult Patch Tests (HRIPT) was performed with 3.75% Galaxolide (65% HHCB in DEP) and a cream control. Nine inductions were made by application of semi-occlusive patches (Webril patch affixed to the centre of a 1x3 inch elastic bandage) containing 0.5 ml of the preparation for 24-hr periods to the upper arms of the subjects, 3 times a week for 3 weeks. After a rest period of two weeks, a 24-hr challenge patch identical to the induction patches was made on a site previously not exposed. Reactions were scored 48 or 72 hr after the challenge patch application. Little or no primary irritation and no allergic potential was evidenced among the 40 subjects tested with Galaxolide (Rubenkoenig and Ede, 1964).

In another HRIPT test, 43 subjects (group 118) were tested with a 50 % solution of neat HHCB in ethanol by application of 0.5 ml of the solution on test patches (1x1 inch Webril swatch affixed to the centre of a 1x2 inch elastic bandage) to the upper arms for 24 hrs 3 time/week for 3 weeks (9 applications) to the same site if possible. Approximately 2 weeks after removal of the final patch, challenge duplicate patches were applied, one to the original site, one to a fresh skin site. Scores were recorded at 48 or 72 hours after application of the patches. HHCB and the solvent control resulted in little or no primary irritation and no sensitisation. (Guillaume, et al., 1973a).

The same authors performed a HRIPT test using the same protocol but with 100% neat HHCB without vehicle on 42 subjects (group 117). Neat HHCB resulted in little or no primary irritation and no sensitisation. (Guillaume, et al., 1973b).

In a limitedly reported study, a human maximization test was performed using Galaxolide 50 (65% HHCB in DEP) probably in petrolatum (which was used as a control). Galaxolide 50 was applied (volume not reported) under occlusion on the volar aspects of the forearm (patch not described) of volunteers on 5 alternate days for 48 hours. Patch sites were pretreated with 5% sodium lauryl sulfate (SLS) under occlusion for 24 hr. to enhance penetration. Challenge patches were applied under occlusion on the back after a 10-14 day rest period with and without pretreatment for 30 min. with 2% SLS. After 48 and 72 hours the sites were scored. Galaxolide demonstrated no potential for irritation or sensitisation in any of the 10 subjects. (Epstein, 1974). Further detail of this study are not available however, it was reported by the Research Institute for Fragrance Materials by a methodology widely recognized at the time for screening of fragrance materials.

In a similar study using Galaxolide 50 with 24 Japanese American subjects, no reactions were found that were considered irritant or allergic. (Epstein, 1979). Further detail of this study are not available however, it was reported by the Research Institute for Fragrance Materials by a methodology widely recognized at the time for screening of fragrance materials.

b) Photosensitisation

A human allergenicity and photoallergenicity study by a modified repeated insult patch test according to the procedure of Kaidbey and Kligman (1980) was conducted in accordance with applicable Good Clinical Practice guidelines. Prior to induction, the minimal erythema dose (MED) for the radiation to be used was determined for each subject. A 25% HHCB (purity not reported) solution in ethanol/diethyl phthalate (75/25) and a vehicle control were applied under occlusive patches along with an untreated patch twice per week for 3 weeks, on the back of 27 panelists. The patch sizes were 4 cm^2 and the aliquot of sample used was 0.2 ml. After 24 hr, the patches were removed and the sites were irradiated with UVB with 5% UVA at approximately 2 times the MED, using a 1000 watt Xenon Arc Solar Simulator with UV-A/UV-B filters (time period not given). Following a 2-week rest period, a single application (identical to that used during induction) of duplicate patches was made to naïve sites. After 24 hr the patches were removed and one of the duplicate patch sites was exposed to 16 J/cm^2 of UV-A and 0.75 × MED of UV-B The test sites were evaluated at approximately 1, 24 and 48 hours following irradiation and non-irradiated patch removal. A total of 27 panelists (6 males, 21 females) completed the study. Slight to one or two strong signs of dermal irritation were obtained during the induction period. However, these reactions were observed in HHCB-treated and vehicle-treated at about the same rate, and even more in blank sites. There was no increase, rather a decrease in the severity of the dermal irritation during the progress of the induction phase. After the challenge, two subjects showed skin responses to the sample, vehicle control as well as blank (untreated site). These reactions are attributed to UV

exposure beyond the subject's ME and are therefore, indicative of a sunburn effect. (Mills, 1997).

Conclusion

Although some questionable elicitation reactions have been reported as a result of patch tests in dermatological clinics on perfume sensitive patients, the available data with guinea pigs and humans (HRIPT and maximisation tests) provide evidence of no potential for induction of sensitisation for HHCB.

There was evidence for no photosensitisation either in a guinea pig test and in a human modified repeat insult patch test at 25%.

Based on all data available HHCB is not considered to be photosensitiser as used in household products.

5.2.1.5 Repeated Dose Toxicity

Oral

A two-week range finding study was conducted in groups of 5 male and female Crl:CD (SD)Br rats receiving HHCB (purity not reported) by dietary admixture at achieved doses of 0, 341, 598, and 679 mg/kg bw/day for males and 0, 352, 633 and 980 mg/kg bw/day for females. In this preliminary study, a progressive dose-related decrease in body weight was observed at the two highest dose-levels in males as well as females. A significantly and dose-related increase in absolute and relative liver weight was reported in males and females at all dose-levels. Histopathology revealed moderate centrilobular hypertrophy in the liver of 1/5 males and 2/5 females in the high dose group. Based on these findings, a 90-day study at 5, 15, 50, 150 mg/kg bw/day was conducted (Api and Ford, 1999, Hopkins, et al., 1996).

A 13-week oral toxicity study in accordance with OECD guideline 408 and conforming to GLP was conducted in 150 Crl:CD (SD)Br rats (5 groups of 15 males (weight 182-260 g) and 15 females (weight152 and 201 g)). They received HHCB (purity not reported in report, confirmed to be >95% pure undiluted material, IFF personal communication with RIFM) by dietary admixture at 0, 5, 15, 50, or 150 mg/kg bw/day. HHCB was added to the diet. Analyses of diet indicated that desired homogeneity was reached. The concentrations of HHCB in the test diets were adjusted weekly based on bodyweight and food consumption from the previous week. The mean achieved daily intakes were 5.4, 15.7, 51.8 and 155.8 mg HHCB/kg bw for males and 5.1, 15.6, 51.9 and 154.6 mg HHCB/kg bw for females. After the treatment period, 3 males and 3 females from the control and the high dose groups were maintained for a treatment-free period of 4 weeks.

Observations included mortality and clinical signs (daily), body weight and food consumption (weekly), ophthalmoscopy (before start, at week 13 and at the end of the treatment-free period, only controls and high dose animals), urinalysis (at week 6 and 12 of treatment and at the end of the treatment-free period), haematology and clinical chemistry (at week 7 and 13 of treatment and at the end of the recovery period), macroscopy, organ weights and histopathology (on all tissues from controls and high dose animals, on all gross lesions, and on lungs, liver, kidneys and male and female reproductive and accessory organs from all animals).

There were no mortalities or adverse clinical signs. Body weight and food consumption of treated groups were similar to those observed in the control group. No changes in

ophthalmologic evaluation were observed and no significant histopathological findings at any dose.

A variety of statistically significant differences between control and test animals were seen in haematology and blood chemistry although these differences were all small, often not proportional to dose, often seen only at one time point and/or in one sex, and, with two exceptions, well within historical controls. This and the fact that these findings were not accompanied by any adverse histopathology leads to the conclusion that they are not adverse effects. There were no significant differences seen at the end of the treatment-free period. No adverse histopathological findings were found in the reproductive organs. (Api and Ford, 1999, Hopkins, et al. 1996).

Dermal

Two subchronic dermal non-GLP studies have been conducted but limitedly reported. In the 13 week study, groups of 15 female rats (Crl:COBS CD (SD) BA strain; weight 156-232 g) were exposed topically unoccluded (gentle inunction to the anterior dorsal shaven skin) to dose levels of 1, 10 and 100 mg Galaxolide (65% HHCB in DEP) /kg bw per day as a 2% (w/v) solution in ethanol. In the 26 week study (with one 13 week interim sacrifice), groups of 20 female rats (Crl:COBS CD (SD) BA strain; weight 156-232 g) were similarly exposed to dose levels of 0, 9, 18 and 36 mg Galaxolide/kg bw/day as a 2% solution in ethanol (area of application not reported). Untreated controls and ethanol controls were included. Observations included mortality, clinical signs, behavioural and motor function and (limited) haematology, serum chemistry, organ weights, macroscopy and histopathology. Special neuropathological examination of brain, spinal cord, and peripheral nerves was included for 2 animals per dose group.

In the 13-week study, there were no reported adverse clinical signs, no variation in biochemistry or haematological parameters, no effects on bodyweight and no histological changes at any dose. However, increased absolute and relative liver weights were seen at 100 mg Galaxolide/kg bw per day, but no actual data were presented so the degree of these changes is unknown. No effects were reported quantitatively in the 26-week study but a decrease in bodyweight was seen with other test materials and it was reported that, “A similar effect was seen at both interim and terminal sacrifice in the 26 week study for animals tested with 36 mg/kg … Galaxolide…” No microscopic changes were observed in the nervous tissues of animals treated with Galaxolide at any of the dose levels employed. These studies were primarily designed to screen for neurotoxicity and a positive control, 7-Acetyl-6-ethyl-1,1,4,4-tetramethyl-1,2,3,4-tetrahydronaphthalene (AETT), a known rat neurotoxin, was similarly dosed at 0.1, 0.3, 1, 3, 10, 30 or 100 mg/kg bw/day for 13 weeks and 3, 18 or 36 mg/kg bw/day for 26 weeks

Clear evidence of neurotoxicity, both clinically and pathologically, was seen with the positive control but no such evidence for HHCB was seen in either study at any dose level. (Gressel, et al., 1980).

In a third non-GLP study also designed to screen for neurotoxic potential, a 10% solution of Galaxolide 50 (65% HHCB in DEP) in 95% ethanol was applied daily unoccluded to groups of 15 male (236-288 g) and 35 or 38 female (178-226 g) Charles River CD rats at doses of 50, 100, 200 mg Galaxolide/kg bw per day for 26 weeks (with 6 and 13 week interim sacrifices). Untreated controls and ethanol controls were included. Haematology, clinical chemistry and urinalysis were performed, organ weights were determined and histopathology was carried

out. Treatment with Galaxolide at 100 or 200 mg/kg bw per day led to the appearance of crusty white or brown material and scabbed areas on the dorsal surface of a few rats. At 200 mg/kg bw male rats showed a trend toward decreased body weight gain early in the study accompanied by a decreased food consumption but the differences were not significant. In this study, oral ingestion or removal by grooming was not prevented so the received doses cannot be determined. Following the same study protocol with doses of 25, 50 or 100 mg AETT (a rat neurotoxin)/kg bw/day showed clear signs of neurotoxicity both in behavioural changes and on microscopic examination. No evidence of neurotoxicity was seen with HHCB at any dose level (Estes, et al., 1980).

However, because 1) neither collars nor occlusion were used to prevent oral intake making it impossible to determine actual exposures, 2) the area of application was not reported, and 3) there was no adverse effect dose, it is impossible to conclude a true NOAEL in terms of dermal toxicity.

Inhalation

A group of 20 female CD rats was exposed by inhalation (whole body) to a fragrance mixture at a nominal concentration of 5 mg/m^3 for 4 hr per day, 5 days per week for 6 weeks (mixture B). A group of 24 female SD rats was exposed to 50 mg/m^3 for 4 hr per day, 5 days per week for 13 weeks (mixture G). These fragrance mixtures were aerosolised with a compressed air nebulizer (particle size was not determined for this particular study, but was assumed to be between 0.5 and 7.5 μm, based on similar studies with 5 other fragrance mixtures). HHCB (purity not reported) was part of these fragrance mixtures, and the level of HHCB to which the animals were exposed was 5.7 μg/m^3 for mixture B and 132 μg/m^3 for mixture G. Exposure to either mixture did not result in mortality, skin reactions or effects on body weight, behaviour or physical appearance, haematology and clinical chemistry, organ weights and gross pathology (including uterus and ovaries), or histopathology (uterus but not ovaries examined) (Fukayama et al., 1999). These studies were not conducted according to GLP. Remark: these study are of limited value because the animals were not exposed to HHCB alone, but to mixtures of fragrances however, this is how exposure to HHCB occurs in reality. In these mixtures HHCB was only present at rather low levels.

Conclusion

In a well-conducted 90-day oral study, a NOAEL of 150 mg/kg bw/day for HHCB in rats can be concluded.

The doses received in the dermal subchronic studies cannot be determined but these studies produced no evidence of neurotoxicity.

When administered as part of a fragrance mixture, inhalation exposure to HHCB up to a maximum tested dose of 132 μg/m^3 for 4 hr per day, 5 days per week for 13 weeks did not result in any toxicity.

5.2.1.6 Genetic Toxicity

Table 5. Genotoxicity studies available for HHCB

Type	Activation	Doses	Results	GLP	OECD	Reference
in vitro Bacterial (*S. typhimurium and E. coli*) Reverse Mutation Assay	with and without S-9	10, 33, 100, 333, 1000, 3333, 5000 ug/plate	negative	Yes	471	Api and San 1999; San, et al., 1994

Type	Activation	Doses	Results	GLP	OECD	Reference
		µg/plate				
in vitro Bacterial (*S. typhimurium)* Reverse Mutation Assay	w & w/o S-9	5, 16.6, 50, 166.6 or 500 µg/plate	negative	No		Mersch-Sunderman 1998a
in vitro Cytogenetic Assay with Chinese Hamster ovary	w & w/o S-9	9, 17, 34 µg/ml & 23, 28, 30 µg/ml	negative	Yes	473	Api and San 1999; Curry & Putman, 1995
in vitro Sister Chromatid Exchange Assay with human lymphocytes	w & w/o S-9	0.025, 0.25, 2.43, 24.25, 48.5, 97 µM	negative	No		Kevekordes 1998
in vitro Unscheduled DNA synthesis with rat hepatocytes	without	0.15, 0.5, 1.5, 5, 15, 50 µg/ml	negative	Yes	482	Api and San 1999; San and Sly, 1994
in vitro Micronucleus Test (human lymphocyte cells)	w & w/o S-9	0.05, 0.49, 4.85, 48.5, 97 or 194 µM	negative	No		Kevekordes 1997
in vitro Micronucleus Test (human hepatoma cells)		0.1, 0.97, 9.7, 97, 194 and 387 µM	negative	No		Kevekordes 1997
in vitro SOS Induction with *E. coli*	w & w/o S-9	0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25 or 50 µg/assay	negative	No		Mersch-Sunderman 1998b
in vivo Mouse Micronucleus Assay		380, 750, 1500 mg/kg	negative	Yes	474	Api and San 1999; Gudi and Ritter, 1997

a) *In vitro*

HHCB (>99% pure) in acetone was tested in the Ames test in absence or presence of Aroclor-induced rat liver S9 at a dose ranging from 10 to 5000 µg/plate according to OECD guideline 471 using *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, TA1538 and *Escherichia Coli* strain WP2 UVRA and appropriate positive controls. Based on preliminary range-finding studies, doses of 10 (not tested in confirmation assay), 33, 100, 333, 1000, 3333 (only tested in confirmation assay) or 5000 µg HHCB/plate were used. Slight precipitation was seen at the three highest doses (≥333 µg/plate). All dose levels of HHCB, acetone (negative control) and positive controls were plated in triplicate. All positive controls gave positive responses to the systems within acceptable ranges. No significant increase in the number of revertant colonies was observed for HHCB at any dose with any of the six strains with or without activation (San, et al., 1994; Api and San, 1999).

A second Ames test was conducted with Galaxolide (65% HHCB in DEP) using *Salmonella typhimurium* strains TA97, TA98, TA100 and TA102 with and without rat liver S-9 (Aroclor 1254-induced) metabolic activation and with appropriate positive controls. The method used resembled OECD guideline 471. The vehicle was DMSO. The doses were 5, 16.6, 50, 166.6 or 500 µg/plate (limit of solubility). All positive controls significantly increased the number of revertants. No significant increase in revertants was seen with HHCB at any dose with or without activation (Mersch–Sundermann, et al., 1998a).

A cytogenetic assay with Chinese Hamster ovary cells (CHO-K_1) was conducted on HHCB (purity >99%) according to OECD Guideline 473. Concentrations of 5, 10 and 20 μg HHCB/ml were used without metabolic activation, using 4/20, 20/20 and 44/44 hr exposure/harvest periods. In the study with metabolic activation (S9 from rat liver induced by Aroclor 1254), dose levels of 8.7, 17.3 and 34.5 μg HHCB/ml were tested for the 4-hr period with a 20-hr harvest time and dose levels of 22.6, 28.2 and 30.0 μg/ml for the 4-hr period with a 44-hr harvest time. At the 20 and 44-hr harvest times, the cells were assessed for structural chromosome aberrations, and at the 44-hr harvest time, also for numerical chromosome aberrations. The mitotic index was significantly lowered at the highest dose in all cases. N-methyl-N'-nitro-N-nitrosoguanidine was used as a positive control in the non-activated study and benzo(a)pyrene in the activated study. Positive controls caused increases in structural (significantly) aberrations in all cases. No significant increase in structural or numerical chromosome aberrations was observed with or without activation with HHCB at any dose. HHCB was concluded to be negative for chromosome aberrations in this test (Curry and Putman, 1995, Api and San, 1999).

The ability of Galaxolide (65% HHCB in DEP) to induce sister-chromatid exchange (SCE) was evaluated using cultured human lymphocytes obtained from healthy non-smoking donors ranging in age from 25-35 years. The method used resembled OECD guideline 479. Cultures were treated with concentrations of 0.025, 0.25, 2.43, 24.25, 48.5 or 97 μM (solvent DMSO) for 2 hours with rat liver S9 activation (Aroclor 1254-induced) or for 24 hours without metabolic activation. After harvest, the cells were scored for SCEs. Cyclophosphamide at 0.1 μM was used as a positive control and produced a significant increase in SCEs Concentrations of HHCB up to 48.5 μM produced no effects (97 μM was too cytotoxic to be evaluated) (Kevekordes, et al., 1998).

An *in vitro* unscheduled DNA synthesis (UDS) assay in accordance with OECD guideline 482 was conducted with HHCB (purity >99%) in acetone in primary rat hepatocytes at concentrations of 0.15, 0.50, 1.5, 5.0, 15 μg/ml (50-5000 μg/ml proved too toxic to test). The positive control 7,12-dimethylbenz(a)anthracene induced a significant increase in the average net nuclear grain count over controls. No increase in net nuclear grain count was seen for HHCB up to and including 15 μg/ml although this dose did induce significant cytotoxicity as determined by LDH leakage. 50 μg/ml proved too toxic to be evaluated. (San and Sly, 1994, Api and San, 1999).

A *in vitro* micronucleus test was conducted with Galaxolide (65% HHCB in DEP) at concentrations of 0.05, 0.49, 4.85, 48.5, 97 or 194 μM using human peripheral lymphocytes cultures obtained from healthy non-smoking donors aged 25-35 years. After induction of mitosis, HHCB (in DMSO) was added to the cultures with and without rat liver S-9 (Aroclor 1254 induced) metabolic activation for 48 hr. After harvest, the cells were scored for micronuclei in binucleated cells. The positive controls (mitomycin –S9, cyclophosphamide +S9) significantly increased the frequency of micronuclei. No significant increase in the frequency of micronuclei was seen with HHCB at concentrations up to 97 μM (194 μM was too cytotoxic to score) (Kevekordes, et al., 1997).

Another *in vitro* micronucleus test was conducted with Galaxolide (65% HHCB in DEP) at concentrations of 0.1, 0.97, 9.7, 97, 194 and 387 μM in DMSO using human hepatoma cells (Hep G2 line), which are capable of some metabolism. After two hr incubation, the cells were harvested and scored for micronuclei in binucleated cells. The positive control cyclophosphamide (1.0 μM) significantly increased the frequency of micronuclei. No

significant increase in the frequency of micronuclei was seen with HHCB up to 194 µM (387 µM was too toxic to score) (Kevekordes, et al., 1997).

An SOS chromotest was conducted by incubating *Escherichia coli* PQ37 with Galaxolide (65% HHCB in DEP) in DMSO at concentrations of 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25 or 50 (limit of solubility in this assay) µg/assay (volume per assay is 310 µl) with and without rat liver S-9 (Aroclor 1254 induced) metabolic activation. 4-Nitroquinoline-N-oxide (-S9) and benzo[a]pyrene (+S9) were used as positive controls. After 2 hr incubation, enzyme activities of β-galactosidase and alkaline phosphatase were measured. Inducing factors, IF, were calculated relative to negative controls (solvent only). Both positive controls significantly increased IF but no inducing potency nor toxicity was seen with HHCB at any dose (Mersch-Sundermann, et al., 1998b).

b) In vivo

In a micronucleus test according to OECD guideline 474, groups of 5 male (28.1-37.2 g) and 5 female ICR mice (24.5-31.0 g) were dosed with 0, 376 750, or 1500 mg/kg bw HHCB (in corn oil - purity >99%) by intraperitoneal injection at a constant volume of 20 ml/kg bw. The high dose was selected to be approximately 70% of the estimated intraperitoneal LD_{50}. The positive control was cyclophosphamide. Bone marrow was harvested at 24, 48 and 72 hr after dosing and examined for micronucleated polychromatic erythrocytes (PCE). No mortality was seen. Lethargy was observed in all animals on 1500 mg/kg bw, in 4/15 males and 4/15 females on 750 mg/kg, and 1/15 males and 0/15 females on 380 mg/kg bw. Moderate reductions (up to 25%) in the ratio of PCE to total erythrocytes were observed in groups on 1500 mg/kg bw after 48 and 72 hrs indicating toxicity and bioavailability to the bone marrow target. The positive control induced a significant increase in micronucleated PCE in both male and female mice at 24 hr (the only harvest time for this group). No significant increase in micronucleated PCE in HHCB-treated groups relative to the respective vehicle control group was observed in male or female mice at 24, 48 or 72 hr after dose administration. (Api and San, 1999, Gudi and Ritter, 1997).

Conclusion

HHCB has been tested in a wide array of well-conducted *in vitro* tests (Bacterial Reverse Mutation Assay (2), cytogenetics assay with Chinese Hamster ovary cells, Sister Chromatid Exchange Assay, Unscheduled DNA synthesis, Micronucleus Tests (2) with human lymphocyte and hepatoma cells and an SOS chromotest) and in an *in vivo* mouse micronucleus test. No evidence for genotoxicity has been seen in any of these tests. It can be concluded that HHCB is a non-genotoxic substance.

5.2.1.7 Carcinogenicity

There are no carcinogenicity data available. HHCB did not show genotoxicity in a battery of genotoxicity tests. There are no indications from repeated dose toxicity studies of potential for carcinogenicity l. HHCB does not have structural alerts for carcinogenicity (Ford, et al., 2000).

Conclusion

HHCB is not considered to have carcinogenic potential.

5.2.1.8 Reproductive Toxicity

No effect on reproductive organs was found in the 13-week oral study after histopathological examination of male and female reproductive and accessory organs from all animals (Lambert and Hopkins, 1996; Ford, 1998). In a peri/postnatal exposure study (see below) no effect on reproduction performance was found (Jones, et al., 1996;Ford and Bottomley, 1997).

Conclusion

There is no evidence that HHCB is a reproductive toxicant.

5.2.1.9 Developmental Toxicity/Teratogenicity

In a study designed to determine the effects of HHCB (purity not reported in report, confirmed to be >95% pure undiluted material, IFF personal communication with RIFM) on the neonate when exposed through nursing, HHCB was administered at dosages of 0, 2, 6 or 20 mg/kg bw/d once daily by gavage in corn oil to groups of 28 time-mated rats (Crl:CD BR VAF/Plus strain) from Day 14 of pregnancy (end of organogenesis) through to weaning on Day 21 post partum. The females were allowed to litter and rear their young to weaning. From these litters, selected offspring were retained (24 males and females per group) to maturity and assessed for behavioural changes and reproductive capacity. The F1 generation was only exposed to HHCB *in utero* during the perinatal phase and through transfer in the milk of the lactating dams. The exposure of the F1 foetuses through mother's milk can be estimated based on a pharmacokinetic study in pregnant/lactating rats (Hawkins, et al., 1996a–See section 5.2.1.9. Animal milk studies). HHCB levels in mother's milk up to 2.3 and 32.8 mg HHCB equivalents (including also metabolites)/l were found at oral doses of 2 and 20 mg 14C-HHCB/kg bw per day, respectively, to the dams (see Table 12). Actual intakes cannot be determined because milk consumption during nursing was not measured.

After parturition, the young were counted, sexed, weighed and examined for external abnormalities. On day 4 *post partum,* the pups were weighed and all litters containing more than 8 pups were culled to 8 retaining, where possible 4 males and 4 females. During the pre-weaning period, all pups were examined to determine the age of reaching certain developmental stages by examining surface righting reflex, startle reflex, air righting reflex and pupil reflex. This F1 generation was also evaluated for behavioural effects by examining changes in motor coordination and balance, activity and avoidance. When the F1 generation reached approximately 84 days of age (having been continuously observed for signs of adverse health) they were mated one male to one female avoiding brother-sister pairings. The females were examined before and after mating to determine time of pregnancy, marked anomalies of the oestrous cycle, median pre-coital time, whether pregnancy had occurred and terminated and duration of pregnancy.

The offspring (F2 generation) were examined for abnormalities at parturition and periodically until day 21 *post partum* at which time the study was terminated.

There were no effects of treatment in any of the treated parent females during pregnancy or lactation. No effects were apparent on development of the F1 generation during the late pre-natal phase, or on postnatal growth, no changes in post weaning behavioural tests or mating performance were seen and post mortem examination of F1 males and females, reproductive capacity, litter data and macroscopic post mortem examination of F2 pups did not reveal abnormalities. The highest dose administered, 20 mg/kg bw/day produced no adverse effects. This study was conducted in accordance with GLP and based on the guidelines endorsed by

the ICH Steering Committee on the Detection of Toxicity to Reproduction for Medicinal Products (Ford and Bottomly, 1997).

A dosage-range finding study was conducted to provide information for the selection of dosages to be used in a developmental study. In this study, groups of 8 pregnant Sprague-Dawley rats were administered HHCB (purity not reported) in corn oil by gavage (5 ml/kg bw/day) at doses of 100, 250, 500 or 1000 mg/kg bw on days 7 through 17 of pregnancy. A control group of 19 pregnant rats received corn oil only. Three rats in the high dose group died on days 10, 10 and 12, respectively. One rat in the highest dose group, found dead on day 12, showed at autopsy six pinpoint dark areas in the fundic mucosa of the stomach. Decreased motor activity, localized alopecia and urine stained fur were observed in dams on 1000 and 500 mg/kg bw per day. In addition, at the 1000 mg/kg bw per day group, red perioral substance, ungroomed coat and changes in defecation (soft or liquid faeces) were observed. Dams on all dose-levels showed reduced body weight gain during the treatment period (day 7-18 of gestation). Dams on 500 and 1000 mg/kg bw showed reduced body weight gain during treatment plus post treatment period (day 7-20 of gestation). Reduced absolute and relative food consumption was seen in dams of all treated groups during treatment period (day 7-18) and during treatment plus post treatment period (day 7-20). Mean foetal body weights were 89.3% of controls at 1000 mg/kg bw. No other treatment related effects were seen (Christian et al., 1997a; 1999)

Based on the range-finding study, HHCB (purity not reported) in corn oil was administered by gavage to groups of 25 female Sprague-Dawley rats on days 7 through 17 of presumed gestation at dosages of 0, 50, 150 and 500 mg/kg bw/day. The dams were observed for signs of toxicity and body weights and feed intake were recorded. On day 20 of gestation, the dams were sacrificed and gross necropsy was performed. The number of corpora lutea in the ovaries were recorded and the uteri were examined for pregnancy, number and distribution of implantations, live and dead foetuses and early and late resorptions and the placenta were examined. All foetuses were weighed and examined for sex and gross external abnormalities. One half of the foetuses in each litter were examined for soft tissue alterations. The remaining foetuses were examined for skeletal alterations.

The 500 mg/kg bw/day dosage group had four to nine ($p \leq 0.01$) rats with excess salivation (9 animals), urine-stained abdominal fur (7 animals), red or brown substance on the forepaws (4 animals) and alopecia (6 animals). Dams on 500 and 150 mg/kg bw/day showed statistically significant dosage-dependent reductions in maternal body weight gains for the entire dosage period (days 7 to 18 gestation). These reductions in weight gain reflected significant weight loss on days 7 to 10 at 500 mg/kg bw per day and significantly reduced weight gains at 150 mg/kg bw/day on days 7 to 10. Weight gains at 150 mg/kg bw per day were comparable to the control group values for the remainder of the dosage period and the post dosage period.

Foetuses in the 500 mg/kg bw/day dosage group showed significantly reduced body weights (litter weights and foetal body weights were decreased) and significant increases in litter and foetal incidences of skeleton (vertebral /rib) variations. In addition significant increases in foetal and litter incidences of incomplete ossification and/or no ossification of sternal centra and a significantly decreased number of ossification sites in the metatarsals were seen at 500 mg/kg bw/day. No other Caesarean-sectioning and litter parameters were affected by administration of HHCB to the dams at doses as high as 500 mg/kg bw/day. The litter averages for corpora lutea, implantations, litter sizes, live/dead foetuses, early and late resorptions, percent resorbed conceptuses and percent live male/female foetuses were

comparable among the four dosage groups and did not differ significantly. No dam had a litter consisting of only resorbed conceptuses and there were no dead foetuses.

Based on a reduction in maternal body weight gains for the dosing period (days 7 to 18 of gestation), the maternal no-observable-adverse effects level (NOAEL) for HHCB was concluded to be 50 mg/kg bw/day. Based on a reduction in foetal body weight, increased incidences of foetal-skeletal (vertebral/rib) malformations, and decreased ossification of sternal centra and metatarsals seen at 500 mg/kg bw, the developmental NOAEL was 150 mg/kg bw/day. This study was conducted in accordance with GLP and evaluated ICH Harmonized Tripartite Guideline stages C and D (Christian, et al., 1997b, 1999).

Conclusion

In an oral peri/postnatal toxicity study (exposure of the F_1-generation to HHCB was only *in utero* during the perinatal phase or through any transfer in the milk of the lactating dams), no toxicity was seen in the dams or their F1 and F2 offspring at dose levels of 2, 6, or 20 mg HHCB/kg bw per day. The exposure of F1 foetuses through mother's milk can be estimated based on a pharmacokinetic study with pregnant/lactating rats given oral doses of 2 and 20 mg 14C-HHCB/kg bw per day. Levels up to 2.3 and 32.8 mg HHCB equivalents (i.e. HHCB + metabolites)/l of whole milk were reported, for maternal oral doses of 2 and 20 mg/kg bw/d, respectively (see Table 12). Actual intakes cannot be determined because milk consumption during nursing was not measured. However, these levels can be compared to the average level of 2.9 ppb with a maximum of 48 ppb in human milk samples (Sonnichsen, et al. 1999).

In an well-performed oral development study there were signs of maternal toxicity at 150 mg/kg bw and higher. There was an increased incidence of variations of the foetuses only at the highest dose of 500 mg/kg bw. The maternal NOAEL is 50 mg/kg bw and the developmental NOAEL is 150 mg/kg bw. HHCB is no more toxic to the foetus than to the dam.

5.2.1.10 Additional data

Endocrine interactions

In a non-GLP study, HHCB in ethanol was added to transiently ERα- or ERβ- transfected human embryonal kidney 293 cells for 24 hr. HHCB weakly stimulated the transcriptional activities (about 6 orders of magnitude less than estradiol) with ERα- but not with ERβ-transfected cells (Seinen, et al., 1999).

In a non-GLP study, HHCB in ethanol (10 mmole/L) was added to estrogen receptor-positive human mammary carcinoma cells (MCF-7) and incubated for 6 days according to the method for the E-screen assay of Soto, et al. (1995). It was tested at 5 different concentrations, the highest being 10 µmole/L with a solvent concentration of 0.1% at the highest. The rate of proliferation of the cells was compared to that of a hormone-free control sample as determined by photometric analysis of the total protein content of the fixed cells. The relative rate of proliferation (test substance relative to control) was then compared to that of 17β-estradiol. HHCB showed a slightly higher but statistically insignificant rate of proliferation relative to the hormone-free control. (Bitsch, et al. 2002).

In a non-GLP study, weanling (21 days old) female Balb/c mice (6 per dose group) were maintained on a diet containing 0, 50 or 300 mg HHCB (purity not reported)/kg diet for 2 weeks. This resulted in mean daily intakes of about 0, 6 or 40 mg/kg bw. At the end of 2

weeks, the mice were sacrificed and uterus, thymus, liver and bodyweights were recorded. Positive control mice were injected with 17β-estradiol on days 1, 5, 9 and 12 of the study. The estradiol treated mice had significantly increased uterine weights and decreased thymus weights. HHCB had no significant effects on body weight or weights of uterus or thymus. Relative liver weights were increased significantly at 50 and 300 mg HHCB/kg of diet (Seinen, et al., 1999).

Conclusion

HHCB has been reported to have a very weak estrogenic potency *in vitro* but such effects are not seen *in vivo.* HHCB is thus not considered to produce endocrine disruption *in vivo*

Toxicokinetics

All studies in this section, with the exception of the analyses of human fat and milk samples (Eschke, et al. 1995 and Rimkus and Wolf, 1996) and the *in vitro* dermal absorption studies with excised rat skin (Ashcroft and Hotchkiss, 1996), followed the basic guidance outlined in OECD Method 417.

Studies in animals

In vivo studies

All available studies in this section were evaluated for information on the absorption, distribution, excretion and metabolism of HHCB in *in vivo* animal studies. Within the subheadings based on the route of exposure, these sections were further subdivided into these fate processes to assist in the evaluation of HHCB in these studies.

Also, additional sections were added to address the available intravenous animal studies, the animal and human milk studies, and the human fat studies.

Dermal

Absorption: In a GLP compliant study, the absorption, distribution and excretion of radioactivity have been determined by topical application (occluded with aluminum foil after evaporation of the solvent (not stated for how long)) with 4.5 mg/kg bw of ^{14}C-HHCB (uniformly labelled in the aromatic ring – radiochemical purity 97.2%) in 200 µl of 70 % aqueous ethanol solution to the shaven backs of 18 male pigmented rats (Lister Hooded, bodyweight *ca* 200g, age 5-7 weeks). The application rate was 0.1 mg/cm^2 over an area of 9 cm^2. (This experiment was conducted for the purpose of obtaining ethical approval for the human simulated exposure experiment (see below) and, thus, skin exposure was limited to 6 hours.) After the 6 hr application the dressing was removed and the remaining dose at the treated area washed off with cotton wool swabs moistened with 70% alcohol. Another occlusive dressing (aluminum foil) was placed on the skin of the animals until sacrifice.

Urine, faeces and expired air were collected for rats killed at 6 hr after start of dosing or later and were analysed for radioactivity and metabolite identification. Pairs of rats were killed at 0.5, 1, 3, 6, 12, 24, 48, 72, and 120 hr after start of dosing. Prior to sacrifice blood was withdrawn for analysis. At sacrifice, tissues (including untreated and treated skin) as well as the remaining carcass were removed for analysis of radioactivity. Urine was collected at 0-6, 6-12 and 12-24 hr and every 24 hr thereafter until 120 hrs. Faeces was collected every 24 hr until 120 hr, and air was collected during 0 – 48 hr post dosing. Average recovery of radiolabel (6 – 120 hr) was 97%.

A majority (mean of 77%) of the applied material remained on the surface of the skin at the time of washing - 6 hr. At that time, about only 3.9% of the applied dose could be detected in excreta and tissues (excluding application site). Although exposure was only for 6 hr, the results indicate that a significant skin reservoir of material (up to approximately 10%) was formed from which material continued to be absorbed up to 120 hr resulting in trace organ levels still being present at this time. Analysis of the dressings applied after surface dose removal, indicated that approximately half of the material in the reservoir was lost to the dressing applied after dose removal. Residual radioactivity in the treated skin declined from 10.54% of the dose at 6 hr to 2.02 % of the dose at 120 hr with a half-life of about 2 days. Based on the amount of radiolabel excreted (see below in excretion section) combined with that remaining in the tissues and carcass, but not including the amount remaining in the skin at the treatment site, at 120 hr (2% of the dose), ~13.7% of the applied dose had been absorbed. (Ford, et al. 1999; Hawkins, et al. 1995).

Distribution: In the rat *in vivo* dermal absorption study described above (Ford, et al. 1999, Hawkins, et al. 1995), plasma levels peaked at about 6 hr (time of removal of dose from surface). Analyses of tissue levels indicated that ~0.7% of the absorbed radiolabel was present at 120 hr; however, ~2% still remained in a skin reservoir at the treatment site at 120 hr. The large majority of the absorbed radiolabel was found in the large and small intestines and their contents (Table 6) consistent with biliary excretion. Organ levels essentially reflected plasma levels only. Fat levels peaked later at 24 hr declining after that. Average recovery of radiolabel (6 – 120 hrs) was 97%. Peak tissue levels of less than 0.2 µg equiv./g tissue were seen generally at 6 hr in adrenals, bone marrow, brain, eye, heart, kidney, lung, lymph node (peak 12 hr), muscle, pancreas (12 hr), skin (12 hr), spleen, testis, thymus, and thyroid.

Excretion: In the rat *in vivo* dermal absorption study (GLP) in rats (6 hr application under occlusion) described above, after 120 hr, 13% of the applied dose had been excreted (primarily in the faeces - 11.6%, with the remainder in the urine - 1.27%, cage wash - 0.08%, and expired air – 0.06%) with the majority excreted within 48 hr (8.3%) (all mean values). No attempt was made to characterize possible metabolites. Average recovery of radiolabel (6 – 120 hrs) was 97% (Ford, et al. 1999, Hawkins, et al. 1995).

Metabolism: No data available.

Table 6 Distribution of radioactivity in selected tissues during 0.5 to 120 hours after dermal application of 14C-HHCB to male rats at a dose of 4.5 mg/kg bw over an area of 9 cm^2 (as µg equivalents/g of tissue) (Ford, et al. 1999; Hawkins, et al. 1995)

Tissues	Time (hr after initial application)								
	0.5	1	3	6	12	24	48	72	120
LI + contents	0.011	0.061	0.48	1.82	8.01	7.74	3.95	2.13	0.67
SI + contents	0.076	0.37	3.31	5.64	5.71	5.07	3.09	1.64	0.56
Stomach + contents	0.033	0.073	0.401	1.11	1.22	0.598	0.810	0.440	0.123
Liver	0.018	0.083	0.213	0.310	0.306	0.228	0.135	0.107	0.053
Fat	0.004	0.023	0.101	0.238	0.367	0.415	0.291	0.242	0.145
Plasma	0.005	0.022	0.054	0.073	0.059	0.046	0.024	0.019	0.009
Adrenal glands	0.028	0.091	0.158	0.161	0.086	0.090	0.043	0.031	0.013

	Time (hr after initial application)								
Tissues	0.5	1	3	6	12	24	48	72	120
Kidneys	0.016	0.063	0.131	0.193	0.130	0.096	0.039	0.028	0.012
Thyroid	nd	0.068	0.108	0.094	0.074	0.043	nd	nd	nd
Untreated Skin	0.001	0.006	0.073	0.019	0.031	0.019	0.010	0.009	0.002

LI = Large intestine, SI = Small intestine

Oral

Absorption: No data available. However, based on similar physical and chemical properties with AHTN, an estimate of 50% absorption after oral ingestion can be made.

Intravenous

Distribution: In a GLP compliant study, groups of four female Sprague Dawley CD rats (bodyweight range 213-230 g – age 10-11 weeks) received a single intravenous administration of 2 mg/kg bw ^{14}C-HHCB (uniformly labelled in the aromatic ring – radiochemical purity 99%) in a 0.4 mg/ml ethanol/Emulphor EL 620/isotonic saline (1:1:7) solution in the tail vein and were sacrificed at 5, 15, 30 min and 1, 2, 4, 6, 12, 24 and 48 hr and 7, 14 and 28 days. Tissues (fat, kidney, liver) were weighed and blood was collected by cardiac puncture. Urine, faeces and air were collected from the 4 animals that were sacrificed at day 7 after every 24 hrs until 168 hours (air up to 48 hrs). The recovery of radioactivity in these 4 animals represented 91.8 % of the dose administered: 89.3% in excreta plus cage washings, 2.14% in the carcass and 0.25% in the liver.

Maximum concentrations of radioactivity were observed in all tissues at 5 min (earliest time of measurement) except for the fat where the maximum was at 2 hr (Table 7). Between 48 hr and 14 days, radioactivity in the plasma and fat decreased with apparent half-lives of elimination of 2.1 and 2.6 days respectively. In the fat, the majority of radioactivity (57-77%) was associated with parent HHCB. In whole blood, concentrations declined between 7 and 28 days with a half-life of 8.5 days with the majority of the radioactivity being associated with the cells while at earlier times it was primarily associated with the plasma. In the kidneys, the decline between 7 and 28 days was with a half-life of 8.6 days (Hawkins, et al. 1997a).

Table 7 Concentrations of radioactivity in tissues after an intravenous doses of ^{14}C- HHCB to rats of 2 mg/kg bw to rats (in µg equivalents/g tissue). (Hawkins, et al. 1997a)

	Tissues				
Time	Plasma	Whole Blood	Liver	Kidney	Fat
5 min	2.57	1.58	8.83	4.65	1.21
15 min	1.94	1.17	5.90	3.11	1.84
30 min	1.54	0.914	4.73	2.38	3.29
1 hr	1.46	0.845	4.03	1.94	5.27
2 hr	1.31	0.716	3.00	1.26	6.64
4 hr	1.06	0.584	2.32	0.914	5.55

Time	Tissues				
	Plasma	Whole Blood	Liver	Kidney	Fat
6 hr	1.04	0.565	2.39	0.817	4.20
12 hr	0.564	0.332	1.99	0.503	4.75
24 hr	0.249	0.148	1.09	0.227	3.66
2 days	0.102	0.0644	0.548	0.0920	2.17
7 days	0.011	0.0108	0.121	0.0237	0.575
14 days	0.00199	0.00438	0.0407	0.00985	0.0989
28 days	0.00050	0.00185	0.0221	0.00415	0.0260

In a GLP compliant study, one male domestic pig (Sus scrofa of Large White Hybrid strain – age 8-12 weeks, bodyweight 33 kg) received a nominal dose of 0.1 mg/kg bw (actual dose 0.101 mg/kg bw) ^{14}C- HHCB (uniformly labelled in the aromatic ring – radiochemical purity >99%) in ethanol/Emulphor EL 620/isotonic saline (1:1:7) solution by intravenous injection into the ear vein. Urine was collected at 0-6 hr and 6-24 hr and every 24 hr up to 14 days and faeces were collected at 24-hr intervals up to 14 days. Blood was collected at 10, 20 and 40 min, 1, 2, 4, 8, 12, 24 hr, 2, 3, 5, 7, 14, 21, and 28 days. Biopsies of skin and underlying fat tissue were taken at 9, 16 and 28 days (day of sacrifice). The recovery of radioactivity via the excreta was 88.1 % of the administered dose. The maximum concentrations of radioactivity in whole blood and plasma were observed at 10 min (earliest collection) (see Table 8).

Table 8 Concentrations of radioactivity in blood and plasma after an intravenous doses of ^{14}C- HHCB to a pig of 0.1 mg/kg bw (in ng equivalents/g). (Hawkins et al. 1997b)

Time (hr)	Whole blood	Plasma
0.17	69.9	108
0.33	60.6	98.2
0.67	50.5	77.7
1	37.5	58.8
2	21.3	34.1
4	11.9	18.2
8	6.8	10.6
12	5.8	8.6
24	3.3	4.9
48	1.8	2.6
72	1.2	1.9
120	0.9	1.4

Time (hr)	Whole blood	Plasma
168	0.7	1.0
336	<0.5	0.5
504	0.4	<0.3
672	<0.3	<0.3

Radioactivity decreased rapidly in blood and plasma during the initial distribution phase with half-lives of ca. 1.1 hr. Thereafter concentrations declined at a slower rate. After 48 hr up to 168 hr the apparent half-life of elimination was about 90-94 hr. At later times (336-672 hrs), concentrations were close to or below the limits of accurate determination. There was no obvious accumulation of radioactivity in blood cells. In fat, the maximal concentration (earliest collection) was at 9 days. After that, the fat concentration decreased slowly and it was < 3.1 ng equiv./g 16 days after injection and < 0.5 ng equiv./g after 28 days. In skin, the maximal concentration (earliest collection) was at 9 days (3.8 ng equiv./g) declining to 0.8 ng eq/g at 16 days and to below the limit of accurate measurement (<0.5 ng eq/g) at 28 days (Hawkins et al. 1997b).

Excretion: In the rat intravenous study (GLP) described above (Hawkins, et al. 1997a), the majority of the radioactivity (53% of the dose via faeces and 23% of the dose via urine) was excreted during the first 72 hr or 48 hr post-dosing for faeces and urine, respectively. Over the entire collection period (168 hr), the excretion via these routes amounted to 61% and 28.1% for faeces and urine. Exhalation of radioactivity could not be detected.

In the pig intravenous study (GLP) described above (Hawkins et al. 1997b), the majority of the radioactivity (63% of the dose via urine and 10% of the dose via faeces) was excreted during the first 48 hr. Over the entire collection period (336 hr) the excretion via these routes amounted to 74% and 14.6 % for urine and faeces. Exhalation of radioactivity was not monitored.

Metabolism: The urine collected from the two (rat and pig) intravenous studies (GLP) described above were analysed for metabolites by Thin Layer Chromatography using several solvent combinations with solvent E (chloroform/methanol/water/formic acid - 75/25/3/3 by volume) giving good separation. Chromatography of urine samples with Solvent E revealed no unchanged HHCB but at least 10 metabolites in the pig and 12 metabolites in the rat (Table 9). Chromatography of urine samples with Solvent H (chloroform/methanol/ammonia 80/20/1 by volume) revealed at least 14 metabolites in pig urine and 10 metabolites in rat urine. None of these metabolites was characterized other than by retention times (R_f).

No change in abundance of any of the metabolites was observed when urine was treated with aryl sulphatase. In contrast, chromatography with Solvent E revealed decreases in the abundance of the 4 of the 5 main pig metabolites (R_f 0.24, 0.33, 0.42 and 0.47) after treatment with β-glucuronidase indicates that these were glucuronide conjugates. Two of these (R_f 0.33 and 0.42) were also seen after similar extraction of the rat urine but only one (R_f 0.42) decreased after treatment, indicating that pig R_f 0.33 is different from rat R_f 0.33. With the exception of one other metabolite in the rat (R_f 0.59), β-glucuronidase treatment caused no significant decreases.

Table 9 Proportion of urinary metabolites as a percentage of dose after intravenous injection in rats or a pig – solvent E (Hawkins, et al. 1998)

R_f value	Pig urine (0-48 hr)		Rat urine (0-24 hr)	
	Untreated	Enzyme treated	Untreated	Enzyme treated
0.12	*	*	0.31	0.25
0.18	*	*	0.96	0.86
0.20	*	*	2.91	3.22
0.24	6.30	1.22	*	*
0.33	10.69	1.28	2.98	3.19
0.42	4.77	0.29	0.43	*
0.47	19.56	0.47	*	*
0.59	0.23	0.40	2.99	0.85
0.63	2.34	6.89	0.52	0.86
0.70	*	*	3.79	3.45
0.74	3.04	14.36	0.5	0.68
0.80	3.45	8.46	*	*
0.83	*	*	0.56	0.93
0.85	6.66	24.9	*	*
0.90	*	1.44	0.89	1.06
0.97	*	*	*	0.46
Others	5.39	2.75	0.72	1.76
Total in urine	62.45		17.56	

*Not detected

Treatment of either pig or rat urine with β-glucuronidase resulted in the increase of the abundance of metabolites that were also detected without enzymatic treatment, except for a pig metabolite with R_f 0.90 and a rat metabolite with R_f 0.97, which were only excreted as conjugates. The principal urinary metabolite in the pig (R_f 0.47) was not seen in the rat nor was the principal metabolite in the rat (R_f 0.70) seen in the pig.

Chromatography with solvent H, revealed 6 metabolites greater than 1% in pig urine, the principal of which (Rf 0.06) decreased significantly on enzyme treatment but the remaining 5 all increased (Table 10). In the rat urine, again the metabolite with Rf 0.06 decreased on enzyme treatment but there were no other significant decreases. Two new metabolites (Rf 0.75 and 0.99) appeared in the enzyme treated sample (Hawkins, et al. 1998). Faecal metabolites were not studied (although in the rat faecal excretion is quite important).

Table 10 Proportion of urinary metabolites as a percentage of dose after intravenous injection in rats or a pig – solvent H (Hawkins, et al. 1998)

R_f value	Pig urine (0-48 hr)		Rat urine (0-24 hr)	
	Untreated	Enzyme treated	Untreated	Enzyme treated
0.06	41.74	4.58	3.89	2.08
0.12	1.40	2.13	2.79	2.93
0.21	0.64	0.89	2.15	2.19

R_f value	Pig urine (0-48 hr)		Rat urine (0-24 hr)	
	Untreated	Enzyme treated	Untreated	Enzyme treated
0.25	0.88	0.28	2.37	2.57
0.34	0.49	0.95	3.62	3.33
0.49	0.66	1.00	*	*
0.52	0.44	0.70	*	*
0.62	1.19	1.74	0.38	0.39
0.69	0.69	2.67	*	*
0.75	3.72	15.09	*	0.69
0.82	2.23	6.61	0.48	0.54
0.88	8.05	24.57	0.24	0.57
0.92	0.10	0.97	1.16	1.28
0.99	*	*	*	0.28
Others	0.22	0.29	0.47	0.71
Total in urine	62.45		17.56	

*Not detected

Animal milk studies

In a GLP compliant study, designed to measure plasma and milk levels that would be reached as a result of oral dosing, ^{14}C- HHCB (uniformly labelled in the aromatic ring – radiolabel purity 98.0%) was administered by gavage to pregnant Charles River CD rats (n=18/group – bodyweights ca 250-500 g – age 10-15 wks) at 2.0 or 20 mg/kg bw as a solution in corn oil (nominal dose levels; actual dose levels were about 10% higher), daily from day 14 of gestation up to 7 days post-parturition. The dosing regimen was designed to achieve steady state prior to parturition but not to have exposure during organogenesis even though it is recognised that some organogenesis occurs after day 14. Milk samples of *ca*. 0.5 ml (after administration of oxytocin) and blood samples of about 4 ml were obtained from 3 dams per dose level at 4, 8 and 24 hr after dosing with HHCB, on days 3 and 7 post-parturition. Milk and blood samples were analysed for radiolabel. In plasma, the highest mean levels of radiolabel were found in the 4 hr samples, declining to about 35% of that level by 24 hr after dosing (see Table 11). Lower levels were consistently seen after 7 days as opposed to 3 days indicating no significant accumulation in plasma. Levels were roughly proportional to dose with levels at 20 mg/kg bw/day approximately 7 fold higher than those at 2 mg/kg bw/day.

Table 11 Analysis of total radioactivity in plasma after daily oral administration of ^{14}C-HHCB in µg equivalents HHCB/ml plasma (Hawkins, et al. 1996a)

Time after parturition	Time after oral administration (hours)	Mean level after oral dose of 2 mg/kg/day	Mean level after oral dose of 20 mg/kg/day
Day 3	4	1.90 ± 0.87	11.08 ± 1.86
	8	1.05 ± 0.43	7.24 ± 0.53
	24	0.33 ± 0.12	2.66 ± 0.62

Time after parturition	Time after oral administration (hours)	Mean level after oral dose of 2 mg/kg/day	Mean level after oral dose of 20 mg/kg/day
Day 7	4	1.21 ± 0.09	8.76 ± 1.57
	8	0.66 ± 0.25	5.06 ± 0.70
	24	0.23 ± 0.05	1.62 ± 0.55

In milk, levels of total residue (Table 12) were also highest at 4 hr after dosing declining significantly by 24 hr. Similar levels were seen after 7 days dosing as compared to after 3 days dosing. Additionally, the major residue in the milk was associated with a peak, which appeared to co-chromatograph with HHCB (the radiolabelled HHCB peak elutes at a retention time of 18-19.5 minutes whereas in the extract, the peak elutes at 18-21 minutes). Although not fully characterised, the HHCB peak in the milk extracts is considered authentic as the metabolites are expected to be more polar and would therefore precede HHCB on the C18 polar column. About 52 - 70% and 47 - 59% of the radioactivity was associated with other materials (metabolites) at the low and high dose, respectively (Hawkins, et al. 1996a).

Table 12 Analysis of total radioactivity and unchanged HHCB in milk after daily oral administration of ^{14}C-HHCB in µg equivalents HHCB/ml milk (ppm) (Hawkins, et al. 1996a)

Milk collection after parturition	Time after oral administration(hours)	2 mg/kg/day			20 mg/kg/day		
		Mean Total radiolabel	HHCB Mean	Ratio HHCB/total residue	Mean Total radiolabel	HHCB Mean	Ratio HHCB/total residue
Day 3	4	1.71 ± 0.20	0.82 ± 0.11	0.48 ± 0.03	32.8 ± 10.9	17.57 ± 6.4	0.53 ± 0.08
	8	0.88 ± 0.20	0.27 ± 0.09	0.32 ± 0.13	12.4 ± 4.4	4.95 ± 1.48	0.41 ± 0.02
	24	0.27 ± 0.11	nd	-	1.69 ± 0.37	nd	-
Day 7	4	2.28 ± 0.66	0.99 ± 0.49	0.41 ± 0.11	25.0 ± 7.0	11.56 ± 4.6	0.45 ± 0.06
	8	1.09 ± 0.20	0.33 ± 0.13	0.30 ± 0.07	16.1 ± 3.55	8.30 ± 2.92	0.52 ± 0.13
	24	0.15 ± 0.03	nd	-	1.34 ± 0.48	nd	-

nd : not detected due to low radioactivity levels

In vitro studies

Absorption: The *in vitro* absorption of ^{14}C-HHCB (position of label not indicated) was measured (non-GLP) using full thickness dorsal skin (male F344 rat) in flow-through diffusion cells. A receptor fluid containing 50% v/v aqueous ethanol to enhance absorption flowed across the underside at a rate of 1.5 ml/hour. Dose solutions of 0.1% and 0.5% in an ethanol/DEP (75:25) vehicle (15 µg/cm^2 and 78 µg/cm^2, respectively) were applied to occluded (Teflon caps) and non-occluded systems. Receptor fluid was collected at 2 hr intervals for up to 72 hr. After 24 or 48 hr radioactivity on the skin, in the skin, in the receptor fluid and in the skin support system was determined by liquid scintillation spectrometry. HHCB was poorly absorbed through non-occluded skin after 24 hr (0.07% of applied dose). Occlusion enhanced absorption at 24 hours to 5.55%. After 24 hr, about 47 or 66% of the applied radioactivity was present in the skin after unoccluded and occluded exposure, respectively. Over 48 hr HHCB continued to be absorbed into the receptor fluid and total

absorption was greatly enhanced by occlusion. No data were presented with respect to the 48-72 hr period. Total recovery of radioactivity was not presented but was stated to be generally > 80% (Ashcroft and Hotchkiss, 1996). These results cannot be used as determinates of dermal absorption because of the use of a non-physiologically relevant receptor fluid, no report of testing the integrity of the skin (the data were taken from a poster presentation) and poor recovery of radiolabel.

Studies in humans

In vivo studies

Human adipose tissue studies

In a study (non-GLP) to measure residues of HHCB, two human fat samples (origin not specified) were extracted with hexane and the extracts analysed by selective ion trap gas chromatography (GC)/mass spectrometry (MS) for residues of HHCB. Residues were found in both samples at levels of 145 and 149 µg/kg fat (Eschke, et al. 1995).

In a similar study (non-GLP), human adipose samples were obtained from 8 females and 6 males in Germany between 1993 and 1995. These samples were extracted with a mixture of water/acetone/petroleum ether and analysed for HHCB residues by GC/mass spectroscopy. HHCB was found in all 14 samples at concentrations ranging from 28 to 189 µg/kg fat (ppb) (mean 82). Although the small number of samples and wide range of data preclude meaningful statistical evaluation, a visual inspection of the data reveals no clear correlation with sex or age (Rimkus and Wolf, 1996).

In a non-GLP study, human fat samples obtained over the years 1983/1984 and –1994 in Switzerland from corpses of 10 females and 5 males (age group 3-100 years) were analysed for residues of HHCB by homogenisation followed by extraction with cyclohexane/ethyl acetate (1:1) and analysed by GC/MS. HHCB was detected in all samples with a range of 12 – 171 µg/kg fat (mean 66 µg/kg) (Müller, et al., 1996).

Human milk studies

In a study (non-GLP) to determine residues of HHCB, two human milk samples (origin not specified) were extracted with hexane and the extracts analysed by GC/MS for residues of HHCB. Residues were found in both samples at levels of 310 and 360 µg/kg fat or 3.3 and 1.5 µg/kg (ppb) whole milk based on measured fat contents of 1.06 and 0.41%, respectively (Eschke, et al. 1995).

In a similar study (non-GLP), five milk samples were obtained from 4 nursing mothers and were extracted according to an AOAC method (Helrich, 1990) and analysed for HHCB residues by selective ion trap GC. All samples contained some HHCB at concentrations ranging from 16 – 108 µg/kg milk fat (Rimkus and Wolf, 1996). The fat contents of these samples were not reported.

In another, larger study (non-GLP) of HHCB residues, milk samples (mean 34 g) were obtained from 107 nursing mothers in Germany (mean age 31.5 years, mean body mass index 24.5 kg/m^2 at time of child birth and 23.2 kg/m^2 at time of milk sampling) under conditions designed to minimize contamination (all equipment was carefully cleaned and the breast area was cleaned 3 times with cotton wool swabs immersed in propylene glycol). All were asked to report on their use of various household products including soaps, detergents and cosmetics

as well as their consumption of fish products. As established in a separate study, the cotton swabs contained 97 to 182 ng HHCB/ml. HHCB was detected at levels up to 1316 µg/kg of fat with a mean of 80 µg/kg. Based on the reported mean fat level of 3.67 %, this corresponds to a maximum level in the whole milk of 48 µg/kg milk (ppb) with a mean of 2.9 ppb.

Higher concentrations of HHCB were seen in subjects with a higher body mass index (BMI), either at parturition or the time of milk sampling. This could suggest that an increased fat storage in the body causes an accumulation of synthetic musk fragrances, which in turn leads to a higher concentration of these substances in the breast milk. If this were true, one would find some positive correlations between the BMI change and/or body weight loss versus musk concentrations in breast milk, however, this turns out to be not the case. Another important biological variable is maternal age, which was found to have no bearing on the musk concentrations in milk fat. There was also no correlation shown with number of siblings, complete time of breast feeding, diet or use of household products and cosmetics (Sönnichsen, et al. 1999).

Dermal

In a GLP compliant study, the absorption and excretion of total radioactivity was determined in 3 human male volunteers. ^{14}C- HHCB (uniformly labelled in the aromatic ring – radiochemical purity 98.3%) was applied to the skin of human volunteers under conditions intended to simulate a typically high exposure from the use of alcohol based products such as perfumes or eaux de toilette, i.e. 0.4% in 70% ethanol. A mean of 1.76 mg ^{14}C- HHCB dissolved in 70 % ethanol (0.48 ml) was applied to 100 cm^2 (0.018 mg/cm^2) area of skin on the upper back. After 30 min to allow the ethanol to evaporate, the area was covered with light gauze dressing. Six hr after application, the dressing was removed and the treated area washed with cotton wool swabs, moistened with 70% ethanol. An area of 6.25 cm^2 was stripped by 5 successive applications of adhesive tape to determine the amount of total radioactivity in the upper level of the horny layer. The treated site was again covered with fresh dressings up to 120 hr after compound application at which time the dressings were taken off and another skin area of 6.25 cm^2 was stripped to determine the remaining total radioactivity in the upper stratum corneum. Samples of blood (at 0.5, 1, 2, 4. 6, 8, 10, 12, 24, 36, 48, 72, 96 and 120 hr) and excreta (urine at 0-2, 2-4, 4-6, 6-12, 12-24, 24-48, 48-72, 72-96 and 96-120 hr intervals, and faeces at 24 hr intervals) were collected during the five-day period.

The majority of the applied material ~56%) was still on the surface of the skin at the time of washing - 6 hr. The first tape stripping at time of removal of the dose indicated that approximately 11% of the applied radioactivity (AR) remained in the upper layers of the stratum corneum. Recovery in the faeces was below the limits of accurate detection (< 0,1 % applied radioactivity, AR) and only one of the three subjects excreted measurable radiolabel in the urine (0.1 % AR). Concentrations in whole blood and plasma were also below the limits of accurate measurement (i.e. < 2.76 ng/ml) at all sampling times. A further 19.5% AR was detected in dressings at the 120 hr time suggesting that considerable radioactivity remained in the skin after washing but was not significantly absorbed. Tape stripping at 120 hr indicated that only trace amounts (0.27% of the dose) remained in the upper layers of the stratum corneum at that time. Assuming that the radioactivity found on the strippings is representative for the entire application site, a total recovery from excreta, dressings, swabs and skin strips of ~ 86.44% AR can be calculated. A separate study indicated that approximately 22% of the HHCB may evaporate under experimental conditions similar to those for this human unoccluded dermal uptake study (Hawkins, et al. 1996b; Ford, et al. 1999).

Oral

No data available.

In vitro studies

The dermal absorption (non-GLP, but with QA statement) of HHCB was determined over a 24-hr period according to the methodology of the SCCNFP. Radiolabeled HHCB (uniformly labelled in the aromatic ring – radiochemical purity 99.3%) was applied in 1% solution in ethanol (96% v/v) to human epidermal membranes (prepared from female breast or abdominal skin and assayed for integrity with tritiated water) supported on a piece of filter paper (for strength) in glass diffusion cells (n=12). The area of the membrane available for absorption was approximately 1 cm^2 and the average applied dose was 20±0.2 $\mu L/cm^2$. The amount of material absorbed into the receptor phase, 6% Volpo N20 (to enhance solubility) in pH 7.4 phosphate buffered saline, after 24 hr was 0.40±0.06% of the applied dose. The majority of applied HHCB (81±2% of the applied dose) was found in the 24-hr surface wipe and donor chamber wash plus wipe. The stratum corneum tape strips contained 5.8±0.8% of the applied dose and the remaining stratum corneum plus epidermis 4.5±0.6% of the applied dose. Levels of HHCB in the remaining stratum corneum plus epidermis, filter paper (on which the epidermis samples rested) and permeated HHCB were combined to produce a total absorbed dose value of 5.2±0.6% of the applied dose. Overall recovery of radioactivity was 92±0.8% (Green and Brain, 2001).

Summary of toxicokinetics, metabolism and distribution

There are no data available on the toxicokinetics of HHCB after oral exposure. Based on analogy to AHTN, a percentage of 50% for oral absorption is taken forward to the risk characterisation.

In the *in vivo* human study under conditions simulating exposure from the fragrance use of HHCB, a 6 hr unoccluded exposure at realistic concentrations in alcoholic solution, resulted in considerable absorption *into* the skin (approximately 20%). However, most of the material in this skin reservoir was not absorbed but was recovered from dressings over the site of exposure over a 120 hr period presumably from reverse diffusion and/or desquamation. Based on amounts excreted, primarily in the urine, approximately 0.1% was actually absorbed under the conditions of this experiment. In addition, because the application was unoccluded about 22% of the applied dose may evaporate under the conditions of the test, which may explain that about 14% of the radioactivity was not recovered in the *in vivo* human dermal absorption study. An *in vivo* study in rats supports the assumption that a good indication of the amount absorbed is the amount excreted.

A similar picture (although, as expected, with considerably higher absorption) was seen *in vivo* with rats where the material was applied for 6 hr under occlusion. Here again, a reservoir in the skin of about 10% of the applied dose was formed after the six-hr application with about 5% of this reservoir being lost presumably from reverse diffusion and/or desquamation to the dressing 120 hr after dose removal. Based on the amount remaining in the tissues, including that at the site of dosing, at sacrifice (2.7%) and the amount excreted (13%) almost all (11.6%) of which was in the faeces, a total absorption under the conditions of this experiment of ~ 16% can be concluded. The principal differences from the human study were

the much larger absorption as a result of the application under occlusion and the well-known fact that rat skin is more permeable than human. For risk characterisation, a value of 16% will be used to estimate the dermal absorption via rat skin. This value also includes the continued absorption from the dermal reservoir at 120 hr (2% of the dose).

The most definitive study of dermal absorption is the *in vitro* absorption study with human epidermal membranes according to the recommendations of the SCCNFP. In this study, 0.4% of the applied dose was found in the receptor fluid after 24-hr, however, 4.5% of the applied dose remained in the epidermis. Adding these amounts to the small amount remaining on the filter paper used to support the membranes leads to the calculation of total absorption of 5.2% of the applied dose. From a separate study, it appeared that under similar conditions about 2.4% of an applied dose might evaporate. Because this study used a 24-hr application and because of the limitations of the human simulated exposure study (primarily the small number of subjects) this figure is used in the risk characterisation.

The intravenous studies in rats and the pig showed that HHCB is rapidly distributed and is excreted primarily in the faeces by the rat as was seen in the dermal study (~ 68% of total excretion as opposed to ~90% after dermal exposure) but in the pig the principle route of exposure is in the urine. In neither of these studies was any evidence of accumulation seen. However, clearance from the fat was considerably slower than from other tissues. It is noteworthy that in neither of these studies was any of the urinary radioactivity present shown to be present as unmetabolised HHCB, however the faeces, which is the major excretion route of the rat, was not analysed for metabolites or parent.

An oral study with pregnant and later lactating rats shows that orally dosed HHCB and HHCB metabolites can end up in the milk. The levels seen in the milk of the lactating dams can aid in the interpretation of the study (see Table 12 above) where neonate rats were exposed to HHCB and its metabolites through nursing.

HHCB is also found in human milk at levels up to 1316 µg/kg fat (equivalent to 48 µg/kg whole milk based on a measured fat content of 3.67%) and in adipose tissue at levels ranging from 12 – 189 µg/kg fat.

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Conclusion

HHCB has been shown to be poorly absorbed dermally and extensively metabolised and excreted. There is no evidence of significant bioaccumulation. For the purpose of risk characterisation, 50% absorption will be used for oral exposure in the animal studies . For dermal absorption of HHCB in rats and humans, values of 16 and 5.2%, respectively, are taken forward to the risk characterisation.

5.2.1.11 Experience with Human Exposure

A study was performed in order investigate the potential of HHCB to elicit potential allergic reactions in sensitive patients. Patch tests were conducted on 179 patients (144 women, 34 men) suffering from dermatitis in which cosmetic allergy was suspected. Exposure to 25% Galaxolide in petrolatum was performed using Silver Patch Testers. Reactions were evaluated after 48 and 72 hr. Positive reactions were observed in 3/179 patients (1.7%), however, the authors note occasional false-positive reactions due to the Excited Skin Syndrome (DeGroot, et al. 1985).

Twenty-eight patients allergic to perfumes and sweet smelling constituents were studied by patch testing in 21 tests recommended by the International Contact Dermatitis Research Group. Galaxolide (purity unknown) was tested at 3% in petrolatum. No sensitisation reactions were observed (Meynadier, et al. 1986).

A multicenter study with patch tests with 48 fragrance materials was reported. Galaxolide 50 (65% HHCB in DEP) was tested in a 1 and 5% solution in petrolatum on 100 patients. The material was applied to the back for 2 days using Finn Chambers on Scanpor tape, and the reactions were evaluated on days 2 and 3 or on days 2 and 4. For Galaxolide 50, no sensitisation was observed. One questionable reaction was noted at 1%. (Frosch, et al. 1995).

No cases of accidental poisoning from exposure to HHCB are known.

5.2.2 Identification of critical endpoints

HHCB has a low acute toxicity either by the oral or dermal route (LD_{50} values >3000 mg/kg). Inhalation is not considered a significant route of exposure.

Given the low total exposure from use or misuse of household cleaning products, acute toxicity of HHCB does not pose a significant risk to the consumer.

HHCB is not a skin or eye irritant and shows no phototoxicity potential in humans at concentrations significantly higher than would be encountered from the use of fragranced consumer products. There is evidence either from animal and human studies of no potential for dermal sensitisation. HHCB shows no photosensitisation potential in humans at concentrations significantly higher than would be encountered from the use of fragranced consumer products.

HHCB shows no significant systemic toxicity after repeated oral dosing (NOAEL>150 mg/kg bw/day). There are data from an oral 90-day study with rats showing that HHCB has no effects on fertility. In an oral developmental toxicity study with rats, developmental toxicity only occurred at maternal toxic dose levels ($NOAEL_{developmental\ toxicity}$ 150 mg/kg bw/day, $NOAEL_{maternal\ toxicity}$ 50 mg/kg bw/day).

In an oral peri/post natal study in which female rats were exposed to HHCB from day 14 of gestation through weaning, there were no effects on the dams at maternal doses of up to 20 mg/kg bw/day nor on the pups which were exposed via the milk during nursing. Measurements of levels of HHCB (excluding its metabolites and the sample below detectable limits at 24 hr after dosing) in the milk of the dams dosed at this level compared to the levels found in humans indicate that the pups in the high dose group were exposed to levels approximately 1700 to 6000 times the mean level and approximately 100 to 360 times the maximum level found in human milk samples (2.9 and 48 ng/kg whole milk, respectively).

HHCB is a non-genotoxic substance. There are no data available on the carcinogenic potential of HHCB. The mutagenicity data and the repeated dose studies with HHCB do not indicate a concern with regard to carcinogenicity nor does HHCB possess any structural features that would raise a concern.

It can be concluded that the use of HHCB in household products will result in no endocrine disruptive effects in humans.

A conservative determination (*in vitro* human skin) dermal absorption study shows a 24 hr absorption of 5.2% of the applied dose. Significantly lower absorption has been demonstrated with human volunteers under simulated exposure conditions. The dermal penetration coefficient calculated from the dermal flux (10.3 μg/cm^2 - which was determined in the *in vitro* dermal penetration study) according to the following algorithm: Kp = dermal flux/(exposure time x concentration of test solution); Kp = (0.0103 mg/cm^2)/(24h x 10 mg/cm^3) is 4.29 x 10^{-5} cm/h.

There are no available oral absorption data, but based on structural analogy to AHTN, absorption of at least 50% can be concluded.

5.2.3 Determination of NOAEL or quantitative evaluation of data

In the available oral subchronic toxicity study in rats, no significant effects were seen at levels up to 150 mg/kg bw/day. Based on the observation of hepatotoxicity at 350 mg/kg bw/day, the NOAEL can be considered to be 150 mg/kg bw/day. The available dermal subchronic studies are not of sufficient quality to determine a NOAEL but clearly demonstrate the lack of neurotoxicity of HHCB. In a rat developmental toxicity study, there was maternal toxicity as evidenced by significant weight loss on days 7 to 10 at 500 mg/kg bw per day and significantly reduced weight gains at 150 mg/kg bw/day on days 7 to 10. Weight gains at 150 mg/kg bw per day were comparable to the control group values for the remainder of the dosage period and the post dosage period. Thus, the maternal no-observable-adverse effects level (NOAEL) for HHCB was concluded to be 50 mg/kg bw. Based on a reduction in foetal body weight, increased incidences of foetal-skeletal (vertebral/rib) malformations, and decreased ossification of sternal centra and metatarsals seen at 500 mg/kg bw, the developmental NOAEL was 150 mg/kg bw.

Because the principal route of exposure to HHCB from its use in household cleaning products is dermal, it is necessary to consider the systemically available dose from both the oral toxicity studies and the dermal consumer exposure. For the dermal exposure, the dermal absorption from an *in vitro* study using human epidermal tissue of 5.2±0.6% of the applied dose can be concluded. There are no data available on the oral absorption of HHCB so using AHTN as a structural analogue, 50% absorption in the animal studies is assumed.

5.3 Risk Assessment

5.3.1 Margin of Exposure Calculation

The lowest NOAEL from repeated dose oral rat studies is 50 mg/kg bw/day. Based on an assumption of 50% absorption, this translates into a systemic NOAEL of 25 mg/kg bw/day. A highly unlikely total consumer exposure (excluding possible intake from fish and drinking water) of 0.07 μg/kg bw/day (this figure is already adjusted for systemic exposure) is estimated based on use of HHCB at the 97.5 percentile level in fragrances used in household products and the worst-case scenario of maximum use of all categories of such products. Comparison of the systemic NOAEL from the animal study with the systemic exposure results in a calculated margin of safety of over 350,000.

While the presence of HHCB in fish is due to all uses (including perfumes and cosmetics) the margin of safety from exposure (1.4 µg/kg bw/day) resulting from consumption of fish can be calculated to be greater than 17,800.

Because HHCB has been found in human milk samples, it is necessary to consider possible risk to the nursing infant from the resulting exposure even though there is no evidence that such occurrence is as a result of the use of household cleaning products. Calculation of exposure to the nursing infant can be calculated to be a mean of 0.35 µg/kg/day with a maximum of 5.8 µg/kg/day. The maximum exposure can be compared to the NOAEL of 150 mg/kg bw/day from the rat 90-day oral study to calculate a MOS of >25,000.

In an oral peri/post natal study in which female rats were exposed to HHCB from day 14 of gestation through weaning, there were no effects on the dams at maternal doses of up to 20 mg/kg bw/day nor on the pups which were exposed via the milk during nursing. Measurements of levels of HHCB (excluding its metabolites and the sample below detectable limits at 24 hr after dosing) in the milk of the dams dosed at this level compared to the levels found in humans indicate that the pups in the high dose group were exposed to levels approximately 1700 to 6000 times the average levels and approximately 100 to 360 times the maximum level found in human milk samples (2.9 and 48 ng/kg whole milk, respectively).

5.3.2 Risk Characterisation

Given the very low exposures from its use in household cleaning products and the resulting very high Margin Of Safety, HHCB presents no significant risk from the normal use or from accidental misuse of these products. The determined MOS is certainly large enough to account for the inherent uncertainty and variability of the hazard data on which it is based. The MOS is based on worst-case exposure assumptions and a well-defined systemic NOAEL. The true consumer exposure is with a very high likelihood significantly lower than presented here.

5.4 Discussions and Conclusions

Consumers are exposed to HHCB as a result of its common use in fragrance oils which are used in laundry and cleaning products. While higher exposures may occur as a result of the use of the fragrance oil in perfumes, cosmetics and other consumer products, the exposure from use of household cleaning products is the only one considered here. This exposure to consumers is mainly via the dermal route and occurs mainly in hand-washed laundry, laundry pre-treatment and hand dishwashing and to a very minor extent also through residues in the fabric after the washing cycle and skin contact during hard surface cleaning. Consumers are orally exposed to HHCB through residues deposited on eating utensils and dishes after hand dishwashing. Since fragrances are also used in spray cleaners, the consumer can also be exposed to HHCB containing aerosols. Because of the very low volatility of HHCB and low levels of use, inhalation exposures are negligible compared to dermal. The consumer aggregate exposure to HHCB from the use of household cleaning products has been estimated to be at maximum 0.07 µg/kg bw/day.

The available toxicological data demonstrate that HHCB is not a skin or eye irritant and shows no phototoxicity potential in humans at concentrations significantly higher than would be encountered from the use of fragranced consumer products. There is significant evidence from animal and human studies of lack of potential for dermal sensitisation. HHCB shows no

photosensitisation potential in humans at concentrations significantly higher than would be encountered from the use of fragranced consumer products. HHCB shows no significant systemic toxicity after repeated oral dosing (NOAEL>150 mg/kg bw/day). There are no indications for effects on fertility in the oral 90-day study with rats. In an oral developmental toxicity study with rats, developmental toxicity only occurred at maternal toxic dose levels ($NOAEL_{developmental\ toxicity}$ 150 mg/kg bw/day, $NOAEL_{maternal\ toxicity}$ 50 mg/kg bw/day).

The comparison of the aggregate exposure and the systemic NOAEL results in a MOE of over 350,000. This is a very large margin of exposure, large enough to account for the inherent uncertainty and variability of the hazard database and inter and intra-species extrapolations, which are usually considered by a factor of 100.

In summary, HHCB does not pose a risk of adverse health effects to consumers from use in household cleaning products. The uses of HHCB in cosmetics have been reviewed by the SCCNFP (SCCNFP, 2002), which concluded “that HHCB can be safely used as a fragrance ingredient in cosmetic products without any restriction for its use.”

6 References

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7 Contributors to this Risk Assessment

This Risk-Assessment has been developed by the European Flavour & Fragrance Association (EFFA) and its consultants. Additional input was provided by the experts of the HERA Human Health Task Force.