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Test Plan for 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-
hexamethylcyclopenta- γ -2-benzopyran
(HHCb) CAS# 1222-05-5

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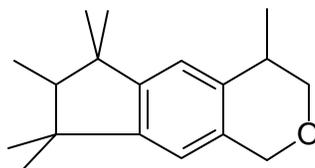
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1 General Substance Information

1.1 Identity of Substances

CAS-No.:	1222-05-5
IUPAC name:	1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta- γ -2-benzopyran (also CAS name)
Synonyms:	1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylindeno(5,6-c)pyran (EINECS name) HHCB Abbalide Chromanolide Pearlide Galaxolide
Molecular formula:	C ₁₈ H ₂₆ O
Structural formula: (main isomer)	



Molecular weight: 258.41

1.2 Introduction

HHCB (1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-gamma-2-benzopyran and related isomers) is used as an ingredient in the fragrance formulations of a wide variety of consumer products ranging from hydroalcoholic (typically in 70% ethanol) type products such as colognes and eau de toilettes to soaps and detergents.

HHCB consists of a main isomer and other minor structurally related isomers. It is a viscous material and is commonly sold and used as an approximately 65% dilution in a neutral solvent. Because this is the primary item of commerce, much of the safety testing has been conducted on the diluted material rather than the pure material.

Trace amounts of HHCB have been reported in human milk and fat samples as well as in the environment (Ford, 1998, Balk and Ford, 1999a). As a result, this material has been thoroughly tested for potential adverse effects in the environment and in humans. The results of these tests have been used to assess the risk to humans under the conditions of use in consumer products (Ford, 1998) and at the levels

found in the environment (Balk and Ford, 1999b). No additional testing of HHCB is anticipated under the HPV challenge program.

2.0 Physical properties

2.1 Melting point

Since HHCB is a mixture of isomers a lower and broader melting point is to be expected. The reported melting point is $-10 - 0^{\circ}\text{C}$ (IFF, 2001).

2.2 Boiling Point

The boiling point of HHCB has been determined by calculation as well as by measurement. The boiling point for HHCB is 160°C at 4 mm Hg as measured during the distillation of HHCB in the manufacturing plant (IFF, 2001). This conforms to the calculated value of 162°C at 4 hPa using the Stein and Brown method.

2.3 Vapor Pressure

The measured vapor pressure for HHCB is 0.0727 Pa at 25°C (Balk and Ford, 1999a).

2.4 Octanol/Water Partition Coefficients

The log Kow value for HHCB was determined according to OECD guideline no. 117 to be 5.9 (Balk and Ford, 1999a).

2.5 Water Solubility

The measured water solubility using ^{14}C -labeled HHCB in three buffered solutions (pH 5, 7 and 9) by the flask method in accordance with OECD protocol 105 was determined to be 1.75 mg/L at 25°C (Balk and Ford, 1999a).

2.6 Relative Density

The relative density of HHCB was determined to be $0.99 - 1.015 \text{ g/cm}^3$ at 20°C using an oscillating densitometer and OECD Method 109 (IFF, 2001).

2.7 Flashpoint

The flashpoint of HHCB was determined to be > 100 °C using the closed cup, Pensky Martens Method (IFF, 2001).

2.8 New Testing Required

No new testing is required.

3 Environmental Fate

3.1 Photodegradation

The photodegradation of HHCB was studied by Aschmann et al. (2001) under laboratory conditions using black lamps for irradiation ($\lambda > 300$ nm) at 25 °C and 740 mm Hg (0.986 bar) total pressure of purified air at ~5% relative humidity. Photolysis and chemical reaction with OH radicals is the dominant atmospheric loss process. The measured rate constant for the gas phase reactions of OH radicals was $k_1 = 2.6 \pm 0.6 * 10^{-11} \text{ cm}^3 \text{ molecule}^{-1}\text{s}^{-1}$, which agrees with the rate constant estimated from the structure of HHCB = $3.8 * 10^{-11} \text{ cm}^3 \text{ molecule}^{-1}\text{s}^{-1}$. Combined with estimated ambient atmospheric concentrations of OH radicals an atmospheric lifetime of 5.3 hours is calculated ($t_{1/2} = 3.7$ h). The calculated lifetimes are inversely proportional to the assumed reactant concentration, and hence the lifetimes depend on time of day, season, and latitude. These data suggest that the atmospheric lifetime of HHCB is sufficiently short that it will not undergo long-range transport to any significant extent (Aschmann et al., 2001).

3.2 Stability in Water

Based on the chemical structure, HHCB is predicted and expected to be unreactive with water. The potential for any significant hydrolysis reaction at the benzylic position is minimized. In order to facilitate hydrolysis of the benzyl ether moiety, either a high concentration of strong aqueous acids or a large quantity of metal salts would be required. Additionally, HHCB has low water solubility, thereby reducing the potential for reaction even further. Thus, there is no significant potential for the hydrolysis of HHCB in water.

3.3 Biodegradation

Biotic degradation

Mineralization

The ready biodegradability of HHCB was assessed in (a) the sealed vessel headspace with total inorganic carbon analysis for CO₂-evolution and an adapted inoculum and (b) the modified Sturm test for CO₂-evolution (Balk and Ford, 1999a). In (a), HHCB was tested as a dilution in isopropyl myristate. The CO₂ evolved during the test was attributed solely to the biodegradation of isopropyl myristate. Test (B)

was conducted on undiluted, viscous HHCB. Biodegradability was not observed. Both tests show the absence of mineralization under the stringent conditions of the tests for ready biodegradability. The tests are summarised in Table 3.3.

Table 3.3. Summary of tests for biodegradation (mineralization)

Modification of OECD 301B , Sealed vessel TIC test acc. to Birch and Fletcher, 1991	
Inoculum	Effluent from SCAS after 8 weeks adaptation
Test substance	HHCB in isopropyl myristate (commercially available quality), 10.97 mg C/l; 32.2%
Dispersion	Injection in isopropyl myristate
Test duration	28 days
Controls	Reference substance benzyl alcohol No toxicity control
Detection	TIC (Total Inorganic Carbon)
Results	% CO ₂ release: zero (corrected for isopropyl myristate)
Modified Sturm test OECD 301B , CO ₂ -evolution	
Inoculum	sewage effluent, 1 drop/l
Test substance	HHCB, nominal 10 and 20 mg/l
Dispersion	No
Test duration	28 days
Controls	Reference substance Sodium benzoate; Toxicity control
Results	% CO ₂ release: zero

Primary degradation

Though not readily biodegradable, HHCB has been demonstrated to degrade in the environment to more polar metabolites, with the lactone and the hydroxycarboxylic acid as likely intermediates. Primary degradation has been demonstrated in soil in the presence of common soil fungi (*Phanerochaete chrysosporium* and *Cladosporium cladosporioides*) (Balk and Ford, 1999a and Van de Plassche and Balk, 1997).

The fate of ¹⁴C-HHCB in soil or sediment was studied in a microcosm study. Samples were taken from an oak forest soil, an agricultural soil and the sediment of the Delaware River in central New Jersey and from a farm with routine sludge applications from a domestic STP in southern New Jersey. Sealed flasks with soil spiked with 10 µg HHCB/g soil were incubated at laboratory ambient temperature for one year. For the four different soil types, an average of 14% HHCB remained after one year. Rate constants were 0.0066 d⁻¹ for sludge-amended soil, 0.0073 d⁻¹ for forest soil, 0.0029 d⁻¹ for agricultural soil and 0.0088 d⁻¹ for river sediment. The estimated half-lives were 105, 95, 239 and 79 days, respectively. The average half-life in the four soils is 128 days (Balk and Ford, 1999a).

¹⁴C-HHCB was dosed at 25 µg/l to activated sludge collected from three different STPs, and to river water (1 µg/l). The disappearance of the parent substance and the formation of metabolites were monitored over time. The half-life for the parent substance in activated sludge was determined to be 21 hours and in river water it was found to be 33 hours.

RP-HPLC analysis of the test media revealed that the metabolites in the activated sludge test (co-eluting with the lactone ($\log K_{ow}$ 4.0) and hydroxycarboxylic acid ($\log K_{ow}$ 0.5) standards) had lower K_{ow} s than the standards: from <0.1 to 3.1. It was suggested that further oxidation of these products had occurred. The capacity to metabolise HHCb was observed in all three STPs included in the study (Langworthy et al, 2000).

3.4 Fugacity

Transport and distribution in the environment were modelled using a Level III Fugacity Model through the EPA EPI Suite 2003 program. The input parameters used were molecular weight (258.41), molecular formula (C₁₈H₂₆O) smiles notation O(CC(c1cc(c2C(C3C)(C)C3(C)C)c2)C)C1, water solubility (1.75 mg/L at 25 degrees C), partition coefficient (Log K 5.9), and vapour pressure (0.0727 Pa @ 25 degrees C).

The model predicts that HHCb is distributed mainly to the sediment (55.6%) and soil (38.6%). The remaining material is distributed to water (5.58%) and air (0.188%).

Sediment concentrations of HHCb have been measured in European rivers. Chronological measurements have indicated a downward trend over time (HLUG, 2001). Primary degradation of HHCb in soil to more polar metabolites has been demonstrated in experiments conducted with soil samples as discussed above (Balk and Ford, 1999a).

3.5 New Testing Required

No new testing is required.

4 Ecotoxicity

All studies in this section have been reviewed and used in environmental risk assessments in two recent publications (Balk and Ford, 1999a and 1999b).

4.1 Acute Toxicity to Fish

A 21-day prolonged toxicity test was carried out on HHCb with bluegill sunfish (*Lepomis macrochirus*) according to OECD Test Guideline 204 under flow-through conditions. The 21-d LC₅₀ was 0.452 mg/l. The overall NOEC of the test was 0.093 mg/l as determined by the onset of clinical signs (Balk and Ford, 1999b; Wuthrich, 1996a).

4.2 Acute Toxicity to Invertebrates

For *Daphnia magna*, a semi-static 21-d toxicity test was carried out with HHCb according to OECD Test Guideline 202, part II, proposed updated version of June 1993 (Balk and Ford, 1999b; Wuthrich,

1996b). Under these conditions, the measured NOEC and LOEC were 0.111 and 0.205 mg/L, respectively and the 48 hr EC50 was 0.28 mg/L.

4.3 Acute Toxicity to Aquatic Plants

The toxicity of HHCB to algae was studied in a static test according to OECD Test Guideline 201 with *Pseudokirchneriella subcapitata*. Under the conditions of this test, the measured NOEC and LOEC were 0.201 and 0.466 mg/l, respectively and the EC50 for biomass production was 0.72 mg/L (Balk and Ford, 1999b; Van Dijk, 1997).

4.4 New Testing Required

No new testing is required.

5 Human Health Data

Most of the data in this section on HHCB have been reviewed and evaluated in a recent publication (Ford, 1998).

5.1 Acute Toxicity

Dermal

HHCB was applied to the skin of groups of 7 albino rabbits at a dose of 5 g/kg bw. The material as tested was a commercial sample and therefore, would have been approximately a 65% solution in a neutral solvent (private communication, IFF). Therefore the corrected dose administered was actually 3.25 g/kg bw. Since there were no deaths at that dose, the dermal LD50 was determined to be >3.25 g/kg bw (Moreno, 1975).

An acute dermal limit test was also conducted on 5 female rats. No deaths were seen throughout the duration of the study. The LD50 was reported to be > 5 g/kg (Ford, 1998).

Oral

HHCB was administered to 10 rats at a dose of 5000 mg/kg bw followed by a 14-day observation. The material as tested was a commercial sample and therefore, would have been approximately a 65% solution in neutral solvent (private communication, IFF). Therefore, the corrected dose administered was actually 3.25 g/kg bw. A single mortality was observed throughout the 14 day observation period. Therefore, the oral LD50 was determined to be >3.25 g/kg bw (Moreno, 1975).

An acute oral limit test was conducted in female rats. Administration was by gavage and the rats were observed for 14 days. One death was seen at a dose of 3.25 g/kg. The LD50 is reported to be greater than 3.25 g/kg (Ford, 1998).

5.2 Genetic Toxicity

5.2.1 In Vitro

HHCB was tested in the Ames test (OECD guideline 471) both in absence and presence of Aroclor-induced rat liver S9 at doses ranging from 10 to 5000 µg/plate using *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, TA1538 and *Escherichia Coli* strain WP2 UVRA. No significant increase in the number of revertant colonies was observed for HHCB at any dose with any of the six tester strains either in the presence or absence of activation (Api and San, 1999).

A second Ames test was conducted with 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. No significant increase in revertants was seen with HHCB at any dose (5-500 ug/plate) with or without activation (Mersch-Sundermann, et al. 1998a).

An *in vitro* micronucleus test was conducted with HHCB in DEP using human peripheral lymphocyte 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 S9 (Aroclor 1254 induced) metabolic activation for 48 hr. 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 HHCB in DEP using human hepatoma cells (Hep G2 line) which are capable of some metabolism. 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 *in vitro* unscheduled DNA synthesis (UDS) assay in accordance with OECD guideline 482 was conducted in primary rat hepatocytes. 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 (50 µg/ml proved too toxic to be evaluated) (Api and San, 1999).

The ability of HHCB 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. Concentrations of HHCB up to 48.5 µM produced no effects (97 µM was too cytotoxic to be evaluated) (Kevekordes, et al. 1998).

A cytogenetic assay with Chinese Hamster ovary cells (CHO-K₁) was conducted according to OECD Guideline 473. The doses tested ranged from 9 – 30 ug/ml. HHCB was concluded to be negative for chromosome aberrations in this test (Api and San, 1999).

An SOS chromotest was conducted by incubating *Escherichia coli* PQ37 with HHCB with and without rat liver S-9 (Aroclor 1254 induced) metabolic activation. After a 2-hr incubation, enzyme activities of β-galactosidase and alkaline phosphatase were measured. Inducing factors, IF, were calculated relative to negative controls (solvent only). The tested doses ranged from 0.39 to 50 ug/assay. Both positive controls significantly increased IF but no inducing potency nor toxicity was seen with HHCB at any dose (Mersch-Sundermann, et al. 1998b).

5.2.2 In Vivo

HHCB was tested in a micronucleus test according to OECD guideline 474. The doses tested were 376, 750, and 1500 mg/kg (Api and San, 1999). 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.

5.3 Repeat Dose Toxicity

A 13-week oral toxicity study in accordance with OECD guideline 408 and conforming to GLP was conducted in 150 rats CD (SD) (4 groups of 15 males and 15 females receiving HHCB by dietary admixture at 5, 15, 50, 150 mg/kg bw/day while a control group (15 males and 15 females) received the normal diet.

There were no mortalities or adverse clinical signs. Body weight and food consumption rates of treated groups were similar to those observed in the control group. No changes in ophthalmologic evaluation or significant histopathological findings were observed at any dose. The LOAEL in the study was based on the 2-week range finding study which was 347 mg/kg bw/day (increased liver weights seen at this dose). The NOAEL was determined to be 150 mg/kg bw/day (Api and Ford, 1999).

5.4 Reproductive Toxicity

HHCB was subjected to a peri- and post-natal development study where HHCB was administered by gavage to pregnant rats at daily doses up to 20 mg/kg bw starting in the third week of pregnancy and continuing through parturition until the weaning of the F1 generation. The F1 generation, which had been exposed to measurable levels in the milk (Hawkins and Ford, 1996), was allowed to grow to maturity and produce a third generation. There were no adverse effects on the F0 dams or the F1 and F2 offspring at the highest dose administered, 20 mg/kg bw/day which is the NOAEL for the study.

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 Bottomley, 1997, Jones et al, 1996).

5.5 Developmental Toxicity

HHCB was subjected to a developmental/teratology study in rats by administration of doses of 50, 150 or 500 mg/kg bw/day on days 7 through 17 of pregnancy. 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 and an increased incidence of foetal skeletal (vertebral/rib) variations, the developmental NOAEL was 150 mg/kg bw.

This study was conducted in accordance with GLP and based on the guidelines endorsed by the ICH Harmonized Tripartite Guideline stages C and D (Christian, et al., 1999)

5.6 New Testing Required

No new testing is required

6 Test Plan Table

Physical-Chemical Properties					
Melting point	Boiling point	Vapor Pressure	Partition Coefficient	Water Solubility	
A	A	A	A	A	
Environmental Fate and Pathways					
Photo-degradation	Stability in water		Biodegradation		Fugacity
A	NA		A		Calc
Ecotoxicity					
Acute Toxicity to Fish		Acute Toxicity to Aquatic Invertebrates		Acute Toxicity to Aquatic Plants	
A		A		A	
Human Health Data					
Acute Toxicity	Genetic Toxicity <i>In Vitro</i>	Genetic Toxicity <i>In Vivo</i>	Repeat Dose Toxicity	Reproductive Toxicity	Developmental Toxicity
A	A	A	A	A	A

Legend

A: End point requirement fulfilled with adequate existing data

NA: Not applicable due to physical/chemical properties

Calc: Endpoint requirement fulfilled based on calculated data

In conclusion, the data set on HHCb is robust and satisfies all the end points under the USEPA High Production Volume requirements. Therefore, no further testing is required on this material.

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