HERA

Human and Environmental Risk Assessment
on ingredients of Household Cleaning Products

AHTN (6-Acetyl-1,1,2,4,4,7-hexamethyltetraline) (CAS 1506-02-1 and 21145-77-7)

Version 2.0
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1 EXECUTIVE SUMMARY

AHTN (6-Acetyl-1,1,2,4,4,7-hexamethyltetraline) is a member of a group of substances used in fragrances and known collectively as the polycyclic musks. It is used to make a fragrance long lasting and to have a positive technical effect on its balance bringing the initial and residual smell into harmony.

AHTN is produced in one plant in The Netherlands in an annual volume of 1000 to 5000 tonnes.

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 AHTN in such preparations is typically at a level of several percent. The principal exposure to AHTN 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

The oral LD_{50} values for AHTN ranged from 570-1377 mg/kg bw. The dermal LD_{50}-values are >5000 mg/kg bw. Inhalation exposure has been estimated to be negligible relative to dermal.

AHTN 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 household products. There is no significant evidence either from animal or human studies of potential for dermal sensitisation. AHTN 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 oral study, a NOAEL of 5 mg/kg bw/day for AHTN in rats can be concluded.

There were no indications of effects on fertility or the developing foetus up to and including 50 mg/kg bw/day, the highest dose tested.

There were no effects on rat pups exposed via the milk during nursing to levels of AHTN over 1000 times the maximum level found in human milk samples.

AHTN is a non-genotoxic substance. The mutagenicity data and the repeated dose studies with AHTN do not indicate a concern with regard to carcinogenicity nor does AHTN 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 total exposure to AHTN from its use in household cleaning products is 0.033 μg/kg bw/day. Comparison of this exposure to the NOAEL results in a calculated margin of safety of at least 75,000 and indicates that there is no significant risk to human health from exposure to AHTN as used in household cleaning products.

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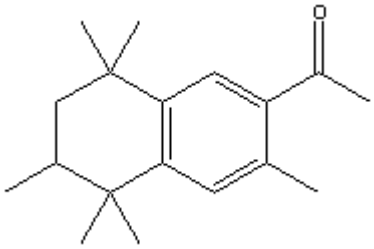
3 SUBSTANCE CHARACTERISATION

3.1 CAS No and grouping information

AHTN (6-Acetyl-1,1,2,4,4,7-hexamethyltetraline) has been registered under two CAS numbers, 1506-02-1 and 21145-77-7. It is a widely used ingredient of fragrance formulations used in soaps, detergents and other cleaning products as well as in cosmetics and fine perfumes. It is also known and marketed as Fixolide, Tentarome, Tetralide, and Tonalid.

3.2 Chemical structure and composition

Molecular description



AHTN

Macro-molecular description

AHTN is a colourless or white crystalline mass or fused opaque mass at room temperature.

Molecular formula/weight

$C_{18}H_{26}O$ – MW 258.41

Melting point

> 54 °C

Boiling point

180 °C at 15 hPa

Vapour pressure at 25° C

0.000682 hPa at 25 °C

Octanol-water partition coefficient

5.7 at 25 °C

Water solubility

1.25 mg/l at 25 °C

Density

0.0006 kg/m3 at 25 °C

Henry's constant

1.393e-4 atm m3/mole (calculated)

3.3 Manufacturing & production/volume

The European Union currently has only one manufacturer of AHTN, PFW Aroma Chemicals BV, which produces AHTN, in an annual volume of 1000 to 5000 tonnes (2001).

Fragrance companies receive AHTN from suppliers in and outside of the EU market or through brokers importing the substance. Fragrance formulations containing AHTN 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 AHTN is as follows: 1) AHTN is manufactured by organic synthesis. 2) Fragrance compounding facilities produce fragrances by blending AHTN with a variety of other fragrance ingredients. 3) These fragrance formulations are used by product manufacturers in various consumer products. The AHTN concentration in the final product is a very small fraction (usually less than 0.1% but always less than 1%). 4) The end products (eg. detergents, household cleaning products and cosmetics) are available for use by consumers worldwide.

Since the synthesis and patenting of AHTN in the 1960s, AHTN has been used as an ingredient in fragrances with a musky scent. Fragrances containing AHTN are used in cosmetic products (per EU's Cosmetic Directive 76/768/EEC and amendments).

3.4 Use applications summary

AHTN 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 AHTN in such preparations is typically at a level of about 1 percent. The principal exposure to AHTN from household products can be considered to be via the skin. The reasonable maximum use levels in household cleaning products ranges from 0.01 to 0.52% (see Table 1 below). 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 Discussion 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 AHTN (6-Acetyl-1,1,2,4,4,7-hexamethyltetraline) as an ingredient of fragrance oils used in household cleaning products. AHTN 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 AHTN can be safely used as a fragrance ingredient in cosmetic products, up to a maximum concentration of 12 % in the fragrance compound."

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 AHTN that are used to formulate these oils in the EU and other geographic locations. Because most household cleaning products are fragranced and AHTN 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 AHTN in consumer 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 AHTN [a]	Level of AHTN in product
Laundry regular powder	0.33	8.7%	0.03%
Laundry liquid	0.80	8.7%	0.07%
Laundry compact (tabs)	0.33	8.7%	0.03%
Laundry compact (powder and other)	0.28	8.7%	0.02%
Laundry liquid concentrate	0.85	8.7%	0.07%
Fabric softener (conditioner)	0.43	8.7%	0.04%
Fabric softener concentrate	0.80	8.7%	0.07%
Laundry additive, powder bleach	0.20	8.7%	0.02%
Laundry additive, liquid bleach	0.20	8.7%	0.02%
Laundry additive, tablet	0.30	8.7%	0.03%
Hand dishwashing liquid	0.23	8.7%	0.02%
Hand dishwashing liquid concentrate	0.45	8.7%	0.04%
Machine dishwashing powder	0.15	8.7%	0.01%
Machine dishwashing liquid	0.15	8.7%	0.01%
Machine dishwashing tablet	0.15	8.7%	0.01%
Surface cleaner liquid	0.60	8.7%	0.05%
Surface cleaner powder	0.25	8.7%	0.02%
Surface cleaner gel	0.75	8.7%	0.07%

Product category	Median use level of fragrance oil in product in %	97.5 percentile use level of AHTN [a]	Level of AHTN in product
Surface cleaner spray	0.13	8.7%	0.01%
Toilet cleaner powder	0.30	8.7%	0.03%
Toilet cleaner liquid	0.35	8.7%	0.03%
Toilet cleaner gel (concentrate)	0.38	8.7%	0.03%
Toilet cleaner tablet	0.30	8.7%	0.03%
Toilet rim block or gel	6.0	8.7%	0.52%

[a]97.5 percentile use level of AHTN 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 AHTN, 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 AHTN 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 AHTN 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 mutually 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 AHTN at the 97.5 percentile use level.

5.1.3.1 Direct skin contact from hand-washed laundry

Hand-washing of laundry is a common consumer habit. During this procedure, the AHTN-containing laundry solution used at an estimated 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 AHTN is different in powder (0.03%) from that in liquid (0.07%) both scenarios are calculated here.

The exposure to AHTN 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	**0.03%** (0.0003) or **0.07%** (0.0007)(Table 1)
C	product concentration in mg/ml:	**10 mg/ml** (AISE/HERA, 2002)
Kp	dermal penetration coefficient	**3.4 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 (8.15 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 = (0.00815 mg/cm^2)/(24h x 10 mg/cm^3) = 3.4 x 10^{-5} cm/h

For powder use:

Exp_{sys} = [0.0003 x (10 mg/ml) x (3.4 x 10^{-5} cm/h) x (0.167h) x 3 x (1980 cm²)] / 60 kg=

0.0017 µg/kg bw/day

For liquid use:

Exp_{sys} = [0.0007 x (10 mg/ml) x (3.4 x 10^{-5} cm/h) x (0.167h) x 1.43 x (1980 cm²)] / 60 kg=

0.0019 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 AHTN 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 AHTN is estimated according to the same algorithm from the HERA guidance document as is used in 5.1.3.1 above using 100% liquid detergent since this contains the highest concentration of AHTN.

F_1	percentage weight fraction of substance in product	**0.07%** (laundry liquid; 0.0007) (Table 1)
C	product concentration in mg/ml:	**1000 mg/ml** (100%)
Kp	dermal penetration coefficient	**3.4 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} = [0.0007 x (1000 mg/ml) x (3.4 x 10^{-5} cm/h) x (0.167h) x (840 cm^2) x 0.5]/ 60 kg=
0.028 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 AHTN exposure from hand dishwashing also uses the algorithm discussed in chapter 5.1.3.1 to calculate the dermal exposure to AHTN 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	**0.02%** (0.0002) (Table 1)
C	product concentration in mg/ml:	**2 mg/ml** (AISE/HERA, 2002)

Kp	dermal penetration coefficient	**3.4 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} = [0.0002 x (2 mg/ml) x (3.4 x 10^{-5} cm/h) x (0.75h) x (1980 cm²) x 3] / 60 kg=
0.001 µg/kg bw/day

5.1.3.5 Direct skin contact from hard surface cleaning

During this procedure, the AHTN-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 AHTN is estimated according to the following algorithm from the HERA guidance document:

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

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) (Table 1)
C	product concentration in mg/ml:	**12 mg/ml** (AISE/HERA, 2002)
Kp	dermal penetration coefficient	**3.4 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	**840cm^2** (TGD, 1996)
n	product use frequency (tasks per day)	**1** (AISE/HERA, 2002)
BW	body weight	**60 kg** (TGD, 1996)

Exp_{sys} = [0.0005 x (12 mg/ml) x (3.4 x 10^{-5} cm/h) x (0.334h) x 1 x (840 cm²)] / 60 kg=
0.001 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 AHTN 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 (containing AHTN) used then the concentration of AHTN in that rinse water can be calculated:

600 ml x 10 mg/ml x 2.5% x 0.07% = 0.105 mg

If 100% is transferred to the 1 kg of fabric, then the concentration in the fabric will be 0.105 mg/kg. Given the fabric density of 10 mg/cm^2 (Procter & Gamble, 1996), it can be calculated that the AHTN is present at 1.05 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 (AHTN) load:	**1.05 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	**4.1%** (0.041) for 24 hr (Green and Brain, 2001)
BW	body weight	**60 kg** (TGD, 1996)

Exp$_{sys\ (indirect\ skin\ contact)}$ = [(1.05 x 10^{-6} mg/cm^2) x (17,600 cm^2) x 0.01 x 1 x 0.041] / 60kg = **1.3 x 10^{-7} µg /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) used for machine laundering. AHTN is present in laundry powder detergents at a maximum level of 0.03% (or 8.1 x 10^{-5} µg AHTN/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 AHTN is estimated to be,

Exp$_{sys\ (inhalation\ of\ detergent\ dust)}$ = [(8.1 x 10^{-5} µg) x 3] / 60 kg = **4.05 x 10^{-6} µg/kg bw/day**

5.1.3.8 Inhalation of aerosols from cleaning sprays

AHTN 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 AHTN-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	***0.01%*** (0.0001) (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)

$$\mathbf{Exp_{sys\ (inhalation\ of\ aerosols)}} = [0.0001 \times (0.35\ mg/m^3) \times (0.8\ m^3/h) \times (0.17\ h) \times 0.75] \ / \ 60\ kg = \mathbf{6.0 \times 10^{-5}\ \mu g/kg\ bw/day}$$

* 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 AHTN

Oral exposures to AHTN 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 AHTN at the limits of detection. Levels in fish have been reported with a 90th percentile level of 0.52 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:

$$\mathbf{Exp_{sys\ (oral\ via\ drinking\ water)}} = [(0.034\ \mu g/l) \times (2L) \times 0.5] \ / \ 60\ kg = \mathbf{0.0005\ \mu g/kg\ bw/day}$$

$$\mathbf{Exp_{sys\ (oral\ via\ fish)}} = [(0.52\ mg/kg) \times (0.115\ kg) \times 0.5) \ / \ 60\ kg = \mathbf{0.5\ \mu g/kg\ bw/day}$$

The daily exposure to AHTN 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 x C` x Ta' x 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	***0.02%*** (0.0002); (Table1)
C`	concentration of product in dish wash solution:	***1 mg/cm^3***
$T_{a'}$	amount of water left on dishes after rinsing	***5.5 x 10^{-5} ml/cm^2*** (Schmitz, 1973)
S_a	area of dishes in daily contact with food	***5400cm^2*** (Official publication French legislation, 1990)
BW	body weight, in kg	***60* kg** (TGD, 1996)

$Exp_{sys\ (oral\ dish\ deposition)}$ = [0.0002 x (1 mg/cm^3) x (5.5 x 10^{-5} ml/cm^2) x (5400 cm^2)]/60 kg = **0.001 µg/kg bw/day**

5.1.3.10 Accidental or intentional overexposure

Accidental or intentional overexposure to AHTN may occur via household detergent products, which may contain up to 0.52% AHTN. 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 0.52% AHTN (this level is seen only in toilet blocks – the highest level in all other household products is 0.07%) the maximum oral exposure resulting from ingestion would be 52 mg. Studies of the acute oral toxicity demonstrate that the toxic dose of AHTN is many times higher than this, even for a small child.

Eye and dermal contact are not considered significant because AHTN is neither an eye nor a skin irritant and the small amount of AHTN 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 sources, the total exposure to AHTN from its use in household cleaning products would be 0.033 µg/kg bw/day excluding possible indirect intake from fish and drinking water. The estimated intake from drinking water is negligible compared to other exposures and the estimated indirect exposure from fish is 0.5 µg/kg bw/day.

5.1.3.12 Special consideration of exposure to nursing infants

Because AHTN 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 AHTN with the use of household products.

In this study a mean level of 16±35 µg AHTN/kg milk fat and a maximum level of 267 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 measured mean fat content was 3.67 % it is possible to calculate the uptake of AHTN via mother's milk as follows:

Mean: 16 µg AHTN/kg milk fat = 16 x 0.120 x 0.0367 = 0.07 µg AHTN/kg bw/day.

Maximum: 267 µg AHTN/kg milk fat = 267 x 0.120 x 0.0367 = 1.2 µg AHTN/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

Groups of five male (bodyweight 245-283 g) and five female (bodyweight 162-187 g) young adult SPF-bred albino rats (Cpb:Wu;Wistar random) were administered by gavage 4.00, 4.80, 5.76, 6.91 or 8.29 ml/kg bw of a 17.5% (w/v) solution of AHTN (Tonalid; purity ≥98%; equivalent to doses of 700, 840, 1008, 1209 and 1451 mg/kg bw AHTN) in isopropyl myristate and observed for 14 days. Within a few hr, signs of sluggishness and piloerection were seen. Later on haematuria, encrustrations around eyes and nostrils and signs of emaciation were observed. Death occurred between 6 hr and 8 days of dosing at which time survivors recovered gradually looking healthy at the end of the observation period. Macroscopic examination at autopsy of the rats that died in the first few days revealed blood stained urine in the bladder. No other treatment-related gross alterations were seen in these or other animals. An LD_{50} of 920 mg/kg bw was calculated (Spanjers and Til, 1985a). This study complied with OECD Guideline 401 except for the variation in volume of dosing.

Groups of five male (bodyweights 242-279 g) and five female (bodyweights 162-186 g) young adult SPF-bred albino rats (Cpb:Wu;Wistar random) were administered by gavage 6.0, 7.2, 8.64, 10.37 or 12.44 ml/kg bw (equivalent to 1050, 1260, 1512, 1815 or 2177 mg/kg bw) of AHTN (Tonalid; purity ≥98%) as a 17.5% (w/v) solution in isopropyl myristate and observed for 14 days. Within a few hr, signs of sluggishness and piloerection were seen. Later on haematuria, encrustrations around eyes and nostrils and signs of emaciation were observed. Death occurred between 6 hr and 8 days of dosing at which time survivors recovered gradually looking healthy at the end of the observation period. Macroscopic examination at autopsy of the rats that died in the first few days, revealed blood stained urine in the bladder. No other treatment-related gross alterations were seen in these or other animals. An LD_{50} of 1150 mg/kg bw was calculated (Spanjers and Til, 1985b). This study complied with OECD Guideline 401 except for the variation in volume of dosing.

Groups of five male (weight 119-133 g) and five female (weight 116-129 g) Sprague Dawley rats were administered by gavage doses of 10 ml AHTN (Fixolide; purity not reported) in 50% ethanol/polyethylene glycol solution with concentrations varied so as to result in doses of 1260, 1588, 2000 or 2520 mg/kg bw and observed for 14 days. The majority of animals

showed lethargy, piloerection, hunched position, oscillated movements, shaggy coat and emaciation. Other occasional signs included green urine, hypothermia, half-closed eyes, difficult breathing and increased breathing, prostration and lacrimation. Upon gross examination alterations were seen in stomach and forestomach, liver and kidneys (discoloration), testes (atrophy) and bladder Deaths occurred on days 5 to 9LD_{50} values of 1331 (males), 1468 (females) and 1377 (combined males and females) mg/kg bw were reported (Meisel, 1982). This study was conducted in accordance with OECD Guideline 401.

AHTN (Fixolide; purity >98%) as a 10% solution in ethanol was administered undiluted (hence, there was variation in volume of dosing) by oral intubation at doses of 0.10, 0.215, 0.464, 1.0 or 2.15 g/kg bw to groups of 5 female Charles River Sprague Dawley rats (initial bodyweight 100–166 grams) that were then observed for mortality and signs of effects for 7 days. Deaths occurred in the high dose group in the first day while in all others occurred during days 2-7. Toxic signs mainly consisted of depression and lethargy. Upon gross necropsy no specific findings were observed, except for three animals in the 1.0 g/kg bw group exhibiting a noticeable yellow coloration of the small and large intestines. An LD_{50} of 0.825 g/kg bw 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 an incompletely reported study, it was reported that oral administration of AHTN (solvent and purity not reported) to groups of ten rats at doses of 0.34, 0.67, 1.31, 2.56 or 5.0 g/kg bw resulted in 100% mortality at 1.31 g/kg bw and higher by day 3. There was one death at the lowest dose and seven at 0.67 g/kg bw. Ataxia was observed from 0.67 mg/kg bw. An LD_{50} of 0.57 g/kg after 14 days of observation was calculated (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 contracted by the Research Institute for Fragrance Materials (RIFM) and was conducted by a standard protocol that was state of the art at the time.

b) Acute Inhalation Toxicity

There are no test data available to evaluate the acute inhalation toxicity of AHTN. Because of the low volatility and low use levels of AHTN, inhalation is not considered a significant exposure pathway compared to the dermal exposure.

c) Acute Dermal Toxicity

AHTN (Fixolide; purity >98%) was administered by inunction to the shaved skin (area not reported) of groups of five female Charles River Sprague Dawley rats (initial bodyweight 108–197 g) in doses of 0.464, 1.0, 2.15 (all 10% solutions in ethanol), 4.64 (20% solution in ethanol) or 10.0 (40% solution in ethanol) g/kg bw that were then observed for 7 days. Toxic signs, mainly seen at the highest dose, consisted of depression, hunching, hind limb weakness and prostration. There were no deaths at any dose on the first day but 0, 1, 0, 1 and 5 deaths were seen at the low to high doses, respectively, during days 2-7, resulting in a calculated dermal LD_{50} of 7.94 g/kg bw. There were no remarkable findings upon gross necropsy (Minner and Foster, 1977). This study was conducted prior to GLP and OECD guidelines but was according to acceptable procedures at the time.

In an incompletely reported study, it was reported that dermal administration of AHTN (solvent and purity not reported) to 10 rabbits at a dose of 5.0 g/kg bw resulted in no deaths after 14 days of observation (Moreno, 1975). Two animals showed anorexia. Signs of skin irritation (slight redness in 3/10 and moderate redness in 7/10 animals, slight oedema in 6/10 and moderate oedema in 3/10 animals) were observed in all animals. The LD_{50} was >5 g/kg bw. This study was conducted prior to GLP and OECD guidelines and specific details

regarding the study are not available. However, the study was contracted by the Research Institute for Fragrance Materials (RIFM) and was conducted by a standard protocol that was state of the art at the time.

d) Acute Toxicity – intraperitoneal

Groups of five female rats (Charles River Sprague Dawley – weighing 104-150 g) were dosed with a 10% solution of AHTN (Fixolide; purity >98%) in ethanol by intraperitoneal injection at doses of 0.0464, 0.10 or 0.215 g/kg bw, and observed for 7 days. In the high dose group 4/5 exhibited depression within 2 hr, 1/5 had died at the end of the first day and all had died by day 3. At the middle dose, animals appeared normal at 2 hr after dosing but slightly depressed at 24 hr. Two were found dead on the 3rd day and 1 more died by day 6. At the low dose, one animal appeared depressed at 2 hr but returned to normal at 24hr; there were no mortalities at this dose. There were no remarkable findings upon gross necropsy. A calculated IP LD_{50} of 0.0794 g/kg bw was reported (Minner and Foster, 1977).

In preliminary studies for a mouse micronucleus study (see section 4.1.2.7 below) groups of 5 male (weight 30-38 g) and 5 female (weight 24.5-29 g) ICR mice were dosed with 26, 100, 300 or 500 (study 1) and 1500, 2500 or 5000 (study 2) mg/kg bw AHTN (in corn oil - purity not reported) by intraperitoneal injection at a constant volume of 20 ml/kg bw and observed for 3 days. In study 1, the mice appeared lethargic at 300 and 500 mg/kg, but there were no deaths. Based on the lack of mortality in study 1, the second study at higher doses was undertaken. In study 2 all mice died within 3 days at the 2 higher doses and signs of lethargy and diarrhoea, but no mortality, were seen in the low dose animals. Based on these findings an IP LD_{50} of approximately 2000 mg/kg was reported (Gudi & Ritter, 1997).

In a study (non-GLP; purity >98%) designed to test the hepatotoxicity of AHTN in rats 6 male Sprague-Dawley rats were treated i.p. once with AHTN in DMSO at 100 mg/kg and sacrificed 7 days later. No death occurred during the 7-day observation period. There was no increase in absolute liver weights but a decreased bodyweight gain compared to untreated controls resulted in increased relative liver weights. Examination of the livers revealed single cell necrosis accompanied by lymphocyte infiltration and Kupffer cell activation. Electron microscopic examination of the livers revealed a strong disruption of the rough endoplasmic reticulum and decreased associated ribosomes. (Steinberg et al. 1999).

Conclusion

The oral LD_{50} values for AHTN ranged from 570-1377 mg/kg bw. The dermal LD_{50}-values are >5000 mg/kg bw.

5.2.1.2 Irritation

a) Skin irritation

In a GLP compliant study according to directive 79/831/EEC, 3 New Zealand White female rabbits were dermally exposed to 0.5 g AHTN (Tonalid; moistened with 0.5 ml water) under a 6.25 cm^2 semiocclusive patch for 4 hr on the dorsal skin (clipped free of fur). Four hours later the treated skin was cleaned by gentle swabbing with cotton wool soaked in warm water. Skin observations were at 1, 24, 48, 72, and 168 hr after patch removal. Controls were not used. No erythema or oedema was seen in any rabbit at any time point. (Haynes, 1984).

In another GLP compliant study according to directive 79/831/EEC, 6 New Zealand White female rabbits were treated dermally with 0.5 ml of a 50% solution of AHTN (Tonalid) in diethyl phthalate (DEP) or with 100% DEP alone (only 4 rabbits). The substances were

applied under a 6.25 cm^2 semiocclusive patch on the dorsal skin (clipped free of fur). Four hours later, the treated skin was cleaned by gentle swabbing with cotton wool soaked in warm water. Skin observations were at 1, 24, 48, 72, and 168 hr after patch removal. For AHTN, after one-hour post exposure, very slight erythema in 4/6 and well-defined erythema in 1/6 was observed. In one of the four animals slight erythema and oedema was seen. At 24 hr, well-defined erythema and slight oedema was seen in 1 rabbit, very slight oedema and erythema in another and very slight erythema in a third animal. Well-defined erythema and slight oedema remained in one animal at 168 hr at which time marked desquamation of the test site was seen. At this time point, desquamation was also observed in another animal in which only slight erythema was observed at the 1 hr and 24 hr observations. Average irritation score for erythema was 0.7 for AHTN treated animals, and 0.2 for DEP treated animals while average scores for oedema were 0.3 for animals with AHTN and 0.0 with DEP (Haynes, 1985).

During the induction phase of a Human Repeat Insult Patch Test (HRIPT) for sensitisation (see below), an occlusive patch (Hilltop chamber 25 mm) with 0.3 ml AHTN (purity not reported) at a concentration of 10% in ethanol/diethyl phthalate (DEP) (75/25) was applied on the backs of 111 subjects for 24 hr three times per week for three weeks. Reactions were scored at 24 and 72 hr after patch removal. Macular erythema was observed at AHTN-treated as well as at vehicle-treated sites at about the same frequencies. Therefore, no irritation was observed after repeated occlusive applications as a result of treatment with AHTN (Berger, 1998).

During the induction phase of an HRIPT, 0.5 ml of 2% AHTN (Tonalid – purity >98%) in dimethyl phthalate was applied on the skin of 52 subjects in patches occluded with impervious tape (area not reported) first on the inner surface of the right deltoid area, then on the inner surface of the left deltoid area, then on the upper portion of the right forearm and then on the upper portion of the left forearm. This cycle was repeated for 10 applications throughout the 23-day induction period. Patches were left on for 48 hr, except for the weekend when they were left in place for 72 hr. Reactions were scored immediately after patch removal. No irritation was observed after repeated occlusive applications (Edelson, 1964). This study is poorly reported.

b) Eye irritation

In a GLP compliant study according to OECD Guideline 405, AHTN (Tonalid 263400 – purity >98%) was applied undiluted as a finely ground powder (0.1 g) to the eyes of 3 male New Zealand White albino rabbits. The test substance remained in the eyes for at least 24 hrs (no further details). Eye irritation was evaluated at 1, 24, 48, 72 and 168 hrs post application. One rabbit developed a slight corneal opacity (score 1 at 24 and 48 hrs, only). Moderate redness and slight to moderate chemosis of the conjunctivae was seen in all three animals after one-hour post application. After 24 hours, this was reduced to slight (1/3 animals) to moderate redness (2/3 animals) and slight chemosis in all three after one-hour post application. After 24 hours, this was reduced to slight (1/3 animals) to moderate redness (2/3 animals) and slight chemosis in all three animals. Complete recovery occurred by 7 days. (Prinsen & van Beek, 1985c).

In a second GLP compliant study according to OECD Guideline 405, AHTN (Tonalid 263406 – purity >98%) was applied undiluted as a finely ground powder (0.1 g) to the eyes of 3 New Zealand White albino rabbits. The test substance remained in the eyes for at least 24 hrs (no further details). Eye irritation was evaluated at 1, 24, 48, 72 and 168 hrs post application. Two rabbits had slight corneal opacity at 24-72 hrs. Slight iritis was observed in one animal at the 1 hr observation and in another animal at the 24 and 48 hrs observations. A slight (1/3

animals) to moderate (2/3 animals) redness and slight (2/3) to moderate (1/3 animals) swelling of the conjunctivae was seen after 24-48 hrs. After 7 days a slight redness of the conjunctivae without any other effects was still seen in two animals. All effects had cleared by 29 days (Prinsen & van Beek, 1985d).

Conclusion

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

5.2.1.3 Phototoxicity

In vivo

Because AHTN 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. In addition, 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.

Because of the number of available studies, they are summarised in Table 2.

Table 2 in vivo animal studies of the phototoxicity of AHTN

Study Type	GLP	Results	Reference
Phototoxicity in guinea pigs	Yes	Positive reactions observed at ≥0.5%	Stern, 1994
Phototoxicity in guinea pigs	No	Positive reactions observed at 1.0%	Klecak et al., 1982
Phototoxicity in guinea pigs	No	Slight reactions observed at ≥0.3%	Klecak and Ciullo, 1983
Phototoxicity in guinea pigs	No	Positive reactions observed at 10%	Guillot, 1985
Phototoxicity – rabbits and guinea pigs	No	Reactions at ≥ 5%	Ogoshi et al., 1980, 1981
Phototoxicity – rabbits	Yes	Negative at 1%	Prinsen & van Beek, 1985b
Phototoxicity – rabbits	Yes	Slight reactions at 2 & 4%	Prinsen & van Beek, 1985a
Phototoxicity –guinea pigs	No	Positive reactions observed at ≥1.0%	Sato et al., 1978
Phototoxicity – hairless mouse	No	Negative at 1%	Forbes et al., 1978a,b
Phototoxicity-human	No	Negative at 10%	Mark and Gabriel, 1987
Phototoxicity-human	No	Negative at 10%	Cuthbert & D'Arcy-Burt, 1983
Phototoxicity-human	No	inconclusive	Folk and Dammers, 1987

Animals

In a GLP compliant study, designed to determine the minimal photoirritation concentration and maximum concentration not producing photoirritation for use in the photosensitization study (see below), photoirritation was assessed by means of visual inspection and by monitoring concentration of extravasal (radiolabeled) albumin in skin biopsies. Groups of 20 female Hartley guinea pigs were i.p injected with 1.0 ml of ^{125}I–bovine serum albumin (^{125}I–BSA; 1 µCi) in a radioisotopic assay before application of 100 µL of a solution of AHTN (according to the study foreword a 1:1 mixture of Tonalid and Fixolide – purity >98%) in absolute ethanol at concentrations of 1.0, 2.0, 4.0 or 8.0% to the shaved and lightly abraded skin (area approximately 1 cm^2). The substance was only applied to one side of the body. The other side of the body was also shaved and lightly abraded, but not treated with AHTN. Approximately 20-30 min after application, for 10 animals per group, the treated and untreated sites were irradiated for 1 hr with broad–band UV–A (7 W/cm^2) and some UV–B light (25-35 µW/cm^2 at 2 cm). Wavelength ranges were not specified. At 4-5 hrs after the application of the test substance or vehicle, the animals were given a second i.p. injection of 1 µCi of radioactive BSA. In a follow-up study, two extra groups of 10 animals were treated with 0.25 or 0.5% solutions in a similar way, but for these dose levels no non-irradiated AHTN-treated controls were used, because no signs of primary dermal irritation was observed in animals treated with 1 to 8% AHTN in absence of irradiation.

In each animal, photoirritancy was evaluated approximately 24 hr after application by comparison of irritation scores for irradiation + AHTN-treated vs. irradiation-only sites. It was stated that no dermal irritation was observed in any site that was not previously treated with AHTN. Irritation of AHTN-treated sites was seen visually (scores higher than vehicle controls) with concentrations as low as 0.5% (not statistically significant) with irritancy scores increasing with concentration (statistically significant at 1% and higher), but only in combination with irradiation.

Using the radioisotopic assay for extravasation (of radioactive BSA), the irritancy response was higher than controls (vehicle + irradiation) at all concentrations tested and also as compared to the AHTN- or vehicle-treated non-irradiated sites, but the response (i.e. the differences between AHTN + irradiation vs AHTN only) was essentially identical at all concentrations. 8- Methoxypsoralen **(**8-MOP) was used as the positive control. According to the foreword of the study, with this substance, photoirritation was observed at concentrations as low as 0.001% or 0.01%, but no further study details or results were presented (Stern, 1994).

In a limitedly reported non-GLP study, AHTN (Fixolide – purity >98%) in ethanol (0.3 or 1%) was applied (dose not reported) to the skin (animal pre-treatment, area size, and patch description not reported) of both flanks of albino guinea pigs for 48 hr. Four hr after patch removal application sites were irradiated with UVA using a Westinghouse FS–40 "Black Lamp" (10^4 erg/cm^2/sec, wavelength: 320-400 nm) or with UVB using Westinghouse FS 40 "Sunlamp" (10^4 erg/cm^2/sec, wavelength: 280-370 nm; for < 15 minutes i.e. less than the minimal erythematous dose). Reactions were measured at 4, 24 and 48 hr after radiation.

Per concentration the following treatment groups were used (each consisting of 10 animals): A: AHTN-treated and irradiated on the right flank with UVA, B: AHTN-treated and irradiated on the left flank with UVB, C: AHTN-treated but not irradiated, D: not treated with AHTN, but irradiated with UVA and UVB.

At 0.3 %, no dermal irritation was observed in any animal, irrespective of treatment with substance or irradiation. At 1 %, all animals of group A showed slight dermal irritation (score 1 in all animals). No signs of dermal irritation were seen in any of the animals in groups B-D. (Klecak et al., 1982)

In another limitedly reported non-GLP study, the phototoxic potential of AHTN (Fixolide NP) was tested according to a CTFA safety testing guideline. AHTN in ethanol at concentrations of 0.3%, 1% or 3% (with 2% DMSO added to enhance skin penetration), was applied to the shaved skin in doses of 25 µL /2 cm^2 area of male or female Himalayan white spotted guinea pigs (4 per test group). Test substances were applied on both flanks of the animals, probably without occlusion. 30 Min after application, the right flanks of the animals were irradiated with a non–erythemogenic dose of UV–A light (20 J/cm^2 over 7 hr, spectrum 320-400 nm). 8-MOP (0.1%) was used as a positive control. No skin reactions were observed on non-irradiated sites, treated with test material or positive control. At 0.3% AHTN, slight dermal reactions (score 1) in 2/4 and in 4/4 animals were observed at 24 and 48 hrs after application, respectively. Both at 1 and 3%, all animals showed slight phototoxic reactions (score 1) at all observation times. With the positive control, slight dermal reactions (score 1) were seen in 4/4 animals at 4 hrs and well-defined dermal reactions (score 2) was observed at 24 and 48 hrs. (Klecak and Ciullo, 1983).

The phototoxic potential of AHTN (purity unspecified) was evaluated in a group (group 2) of 10 male and 10 female young adult albino Dunkin-Hartley guinea pigs in a study conducted to develop a new method for screening for phototoxicity. The animal's fur on back and flanks was clipped and depilated, 24 hr prior to dosing. A single application of a 10% solution of AHTN 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 under an occlusive aluminum foil sheet of 5 × 5 cm. Another (non-treated) part of the dorsal skin was also covered with aluminum foil to protect it from unwanted irradiation.. Five guinea pigs were maintained as a control group (group 1) and were treated with the same solution of AHTN but were not irradiated. The treated patches were irradiated 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 as well as histopathological examinations 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 for the group 2 animals were considered positive if it was 2 units greater than the one attributed to the group 1 animals. Negative and doubtful scores were equal to that or slightly higher than that in group 1, respectively. Positive histopathological readings were those representing "skin 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 8/20 animals, respectively, a positive or a doubtful response was observed, which were confirmed as positive by histopathology. (Guillot, et al., 1985).

(The following was taken from a symposium paper presented in 1980 and from the published paper, in Japanese, presumably reflecting the same data.) A solution of AHTN (Tonalid – purity ≥98%)) at a concentration of 1, 5, 10 or 20% in either petrolatum or 99.5 % ethanol 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 exposed to 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 1 %. Phototoxicity was reported at all higher doses in both species. No data were given at longer periods after irradiation nor were any quantitative data given on strength of the reactions (only -, ± or +) therefore dose response was impossible to determine (Ogoshi, et al., 1980, 1981).

In a GLP compliant study, 0.1 ml of a 1% solution of AHTN (Tonalid –purity ≥98%) in absolute ethanol was applied to the clipped skin (area not specified) of 6 male New Zealand White albino rabbits and the site irradiated with UV light (Philips TL 40W/08 – wave length about 365 nm) for 60 min at 8 inches from the skin surface. A solvent control and a positive control 8-MOP (0.0078% in ethanol) were included in the test. Skin reactions were evaluated at 24, 48, 72 and 96 hr after application and compared to reactions of treated but non-irradiated sites on the opposite flank of the body. In one animal very slight erythema (score 1 at 24 hr, only) was seen after treatment with AHTN, but with and without irradiation. Moderate to severe reactions were seen with 8-MOP with but not without irradiation (Prinsen & van Beek., 1985b).

The above test was then repeated (still GLP) with 2 and 4% solutions in ethanol again with 8-MOP as the positive control. Severe reactions were seen with 8-MOP with irradiation but no reactions without. No significant reactions were seen at either concentration of AHTN without irradiation but both concentrations produced very slight to moderate oedema and very slight erythema with irradiation Primary irritation scores were 1.5 and 1.8 at 24 hr for the 2% and 4% solutions, respectively. The skin reactions decreased in severity over the next 72 hr. The difference in the strength of the reactions seen at 2 and 4% was not considered significant (Prinsen & van Beek., 1985a).

In tests for photoirritation, 0.02 ml aliquots of AHTN (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 0.5, 1.0, 2.5 % in ethanol, 5% in ethanol and DEP, 20% in DEP and 50 % in DEP and ethanol of AHTN (the guinea pigs were not treated with 0.5 and 2.5% and rabbits were not treated with the 50% concentration), followed (time after dosing not specified) by glass-filtered UV-A irradiation for 110 min from 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 the 0.5% with rabbits. Positive reactions (average scores of 1.7 to 2.0 at 24 hr) were seen in guinea pigs from 1 up to 20% compared to scores for the non-irradiated control sites of 0.0. At 50% scores were 2.3-3.0 compared to controls with 1.3-2.3 for the non-irradiated controls over the entire observation period. In the rabbit clear phototoxicity was seen at 5% in ethanol (2.0-3.3) compared to 0.0 in controls) and at 20% in DEP (1.3-3.7 compared to 0.0 in controls). According to the study report, the differences in scores should be considered as an indication of moderate phototoxicity. The average irritation scores declined during the 72 hr period following the irradiation. No positive control was tested (Sato et al., 1978).

An aliquot of 20 µl of a 1% solution of AHTN (Tonalid – purity ≥98%) in methanol 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 sun light using a filtered (Schott WG 320) Osram XBF 6000 w Xenon lamp to produce a very slight erythema, or was irradiated for 1 hr with a bank of F40T12BL fluorescent black lights, (glass-filtered to eliminate "sunburning UV light" (< 320 nm)). Skin reactions were assessed at 2, 4, 24, 48 and 72 hr. No reactions were observed, but irritation scores were not provided (Forbes et al., 1978a).

Forbes et al (1978b) have also studied phototoxicity of AHTN after four applications of 20 µl of 1% AHTN on the skin of hairless mice. This study was stated to be negative, but the report was too incomplete to be taken into account.

Human

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.

AHTN (Tonalid – purity >98% - 10% dilution in ethanol/DEP (3:1)) was tested for phototoxicity on 10 female human volunteers (white Caucasian; ages 18-39). 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 were stripped 3 times to remove the superficial stratum corneum. This was followed by application of 20 µL of the test solution to 3 designated test sites, each approximately 1.5 cm in diameter. One of the sites was used as the non-irradiated control site and one site was irradiated but was not treated with AHTN. 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 contact, one AHTN-treated site and the site not treated with AHTN were irradiated with 10 MED of UV-A followed by 0.5 MED of UVA + UVB The sites were scored 5 min after irradiation and again at 3, 24, 48 and 73 hr after irradiation. There were no significant reactions to the AHTN solution but all three subjects exposed to 8-MOP showed clear positive reactions (Mark and Gabriel, 1987). Twenty five microliters of 1%, 3% or 10% solutions of AHTN (”Fixolide; G9499”) dissolved in ethanol/acetone 1/1 were applied to 6 sites (2 cm^2) per concentration on the back of 6 female volunteers (aged 20-40 yr), after 30 min followed by irradiation with UVA light at a doses of 0, 1, 2.5, 5, 10 or 20 J/cm^2 Skin reactions were compared with those elicited by a positive control (8-MOP 0.005 or 0.01 %, irradiated with 1 and 2.5 J/cm^2) using black light fluorescent tubes having a wavelength of 320–400 nm. Examination of test sites at 4, 24, 48 and 72 hr after application revealed no phototoxic reactions at any concentration of AHTN but slight to severe reactions with 8-MOP (Cuthbert & D'Arcy-Burt, 1983).

In another phototoxicity study, 26 volunteers (male and female Caucasians) received single applications in duplicate of ~0.3 ml AHTN (10% Tonalid in unspecified solvent) under occlusive patches along with 4 other substances and a blank control patch. The test material was applied on the back for 24 hr. The test site was irradiated with 16-20 J/cm² 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. One test material, Tagetes absolute, produced strong phototoxic reactions, which spilled over onto other sites including the blank control making interpretation difficult (Folk and Dammers, 1987). In three volunteers low score responses were observed in non-irradiated patches. In one of these the response increased after irradiation, while in another one, the response did not change after irradiation. In the third one the irradiated site did not show a reaction, but in three additional volunteers low score reactions were observed after irradiation, but not without irradiation. Because of lack of information with respect to the scoring system, these results are difficult to interpret. Data on reactions to the solvent control were not provided. The test is considered inconclusive (Folk and Dammers, 1987).

In vitro

In a GLP compliant study according to the draft OECD guideline/adopted EU-SCCNFP guideline mentioned above, Balb/c 3T3 mouse fibroblasts were exposed to 50 µL aliquots of AHTN (purity >98%) in Hank’s Balanced Salt Solution (HBSS) containing 0.5% ethanol (concentrations of 0.992 – 56.2 µg/ml for irradiated cells or 1.77-100 µg/ml for non-irradiated cells both spaced at a ratio of 1.78) for 1 hr followed by irradiation with UV-A 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 (2 runs) IC_{50} for AHTN was 5.43 µg/ml with irradiation and 6.89 without. The MPE was 0.002 and 0.014 (<0.1 is considered non-phototoxic) for each run and the PIF was 0.949 and 1.233 (>5.0 is considered phototoxic). Chlorpromazine was tested as a positive control. The average IC_{50} was 31.5 µg/ml with irradiation and 2.0 without. The MPE was 0.627 and 0.642 for each run and the PIF was 17.48 and 13.62 (Harbell et al. 2001).

A 1% solution of AHTN (Tonalid – purity >98%) in methanol was applied to filter paper disks, allowed to dry and placed on culture media that had been seeded with yeast (*Saccharomyces*). The plates were then incubated for 24 hr under black light (10 W/m²) and then cultures were examined for growth at 24-hr intervals for 4 days. Growth was compared to that observed in substance-treated plates that did not receive irradiation. Growth inhibition was seen in the area adjacent to the plate and considered as evidence for phototoxicity (Forbes et al., 1978a).

Conclusion

After dermal application, AHTN is photoirritant to guinea pigs and rabbits, although the latter seem to be slightly less sensitive, with a no effect concentration around 0.3-0.5%. It was not photoirritant at 1% in the hairless mouse nor was it photoirritant at 10% on humans in 3 separate studies or in the only recognized *in vitro* photoirritancy test. Based on all data available AHTN is not considered to be photoirritant as used in household products.

5.2.1.4 Sensitisation including photosensitisation

a) Sensitisation

Solutions of 3, 10 or 30% (all of them clearly irritating concentrations under the conditions of repeated application) AHTN (Fixolide – purity >98%) in ethanol/acetone (50/50) were applied in 0.1 ml doses to 8 cm^2 clipped flank skin of 6 guinea pigs each daily for 3 weeks in an open epicutaneous test. Because of cumulative irritation at the site of application especially at the high dose, the application site was changed after 2 wks. Challenge doses of 0.025 ml of a 1% solution (the maximal non-irritating dose in a pre-test) were then applied to a 2 cm^2 naïve site and again 2 weeks later. Reactions are read 24, 48 and 72 hr after each challenge dose, and compared to reactions in non-induced control animals. No sensitisation reactions were observed (Geleick et al., 1978).

In an incompletely reported study, 0.5 ml of a 5% solution (solvent not reported) of AHTN (purity as described in chapter 1) was mixed with an equal volume of Freund's complete adjuvant (FCA) and 0.1 ml injected intradermally into the necks of 8 guinea pigs on days 0, 2, 4, 7 and 9. Control animals received FCA only. Challenge tests of 0.025 ml on a 2 cm^2 skin area (preparation not reported) were then applied 3 and 5 wks after the first intradermal dose and reactions read at 24 48 and 72 hr. It was concluded that AHTN was a weak intradermal sensitiser under these conditions but it was not clear that the reactions reported, 6/8 at day 21 and 8/8 at day 35 were reactions on test or control animals nor were any reaction scores reported (Hoffmann-La Roche, 1978).

A Human Repeated Insult Patch Test (HRIPT) was performed with a 10% solution of AHTN (purity not specified) in ethanol/DEP (75/25) on 111 subjects. Nine induction applications on the same site on the back were made with occlusive patches (Hill Top Chambers, 25 mm) containing 0.3 ml of the preparation for 24 hr-periods, 3 times a week for 3 weeks. After a rest period of about two weeks a challenge application of the same type and duration was made on a site previously not exposed. Reactions were scored 48 and 96 hr after the application of the challenge dose. A mild skin reaction (score 1) was observed 9 persons after the challenge with AHTN, while in 7 persons a similar mild skin reaction was observed with the vehicle. It was concluded that no evidence of allergic potential was seen (Berger, 1998). This test was conducted in accordance with Good Clinical Practice guidelines.

A HRIPT (Tonalid – purity >98%) with a 2% solution of AHTN in dimethyl phthalate was conducted on 52 subjects (age 16-77 yr – 21 M and 31 F) with application of 0.5 ml on patches occluded with impervious tape (area not reported) first on the inner surface of the right deltoid area, then on the inner surface of the left deltoid area, then on the upper portion of the right forearm and then on the upper portion of the left forearm. This cycle was repeated for 10 applications throughout the 23-day induction period. Patches were left on for 48 hr, except for the weekend when they were left in place for 72 hr. After a 2-week rest period, another 48 hr patch of test solution was applied to a naïve site and scoring of reactions was on patch removal and 72 hr later. No irritation or sensitisation reactions were observed on any of the 52 subjects. The study was poorly reported (Edelson, 1964).

A human maximization test was performed with AHTN (2% Tonalid; presumably in petrolatum) of 25 subjects. AHTN was applied (volume not reported) on the volar aspects of the forearm under occlusion (patch not described) of volunteers for 5 alternate day 48 hr periods. Patch sites were pretreated with 5% sodium lauryl sulfate (SLS) under occlusion for 24 hr to enhance penetration prior to the initial patch only. Challenge patches were applied on the back under occlusion (sites pretreated with 2% SLS for 30 min) for 48 hr after a 2-week rest period and the sites scored for reactions 24 and 72 hr after application. No sensitisation reactions were seen. The study was poorly reported (Epstein. 1975).

b) Photosensitisation

M any of the following studies were conducted as method development rather than definitive studies. A summary of available studies is shown in Table 3.

Table 3 Photosensitisation studies with AHTN

species	GLP	Results	Reference
Guinea pig	No	Positive reactions at 1%	Klecak and Ciullo,, 1984a
Guinea pig	No	Positive reactions at 10%	Klecak and Ciullo, 1984b
Guinea pig photosensitisation	No	Positive at 5% with sensitisation reactions to photodegradation products	Klecak and Ciullo, 1985
Guinea pig photosensitisation	Yes	Positive at 10%	Klecak, 1990
Guinea pig photosensitisation	Yes	Positive at 1% induction (slightly abraded skin)	Stern, 1994
Guinea pig photosensitisation	Yes	Negative at 2%	Prinsen & Til, 1985a
Guinea pig photosensitisation	Yes	Negative at 2%	Prinsen & Til, 1985b
Human photo-RIPT	No	Negative at 1 and 5%	Frentzko & Shanahan, 1989
Human photo-RIPT	Yes*	Negative at 10%	Mills, 1997a

species	GLP	Results	Reference
Human photo-RIPT	Yes*	Negative at 10%	Mills, 1997b

*Good Clinical Practice Guidelines

In a photosensitisation assay according to CTFA guidelines, the shaved nuchal area of 10 Himalayan white spotted guinea pigs was treated with AHTN ("Fixolide NP") at a concentration of 10% in ethanol in a dose of 0.1 ml over an area of 8 cm^2 (0.0125 ml/cm^2) and the site irradiated after 30 min with 10 J/cm^2 UVA from a bank of 4 black light florescence tubes. Application of AHTN and exposure to UV light was repeated for a total of 5 times over a 10-day period. One week later, the guinea pigs were freshly shaven on both flanks and challenged with applications of 25 µl of 0.3 % AHTN in ethanol on 8 patches of 2 cm^2 followed by irradiation in the same manner on one site only. Reactions were recorded 24 and 48 hr later and compared to those obtained from a control group of untreated animals. A 3% solution of 3,3',4',5-tetrachlorasalicylanilide (TCSA) in acetone was included as a positive control. Six of the ten guinea pigs were sensitised under these conditions (Klecak and Ciullo, , 1984a).

In another phototoxicity study, apparently similar to the above, a group of 10 guinea pigs (strain not reported) was treated with AHTN (Fixolide NP) as a 1% solution in ethanol for induction as above and challenged with 0.3% in ethanol 1 week later. A 3% solution of 3,3',4',5-tetrachlorasalicylanilide (TCSA) in acetone was included as a positive control. All animals were concluded to be photosensitised under these conditions (Klecak and Ciullo, 1984b).

A group of 10 guinea pigs were photosensitised to a 5% ethanolic solution of AHTN (Fixolide NP – purity >98%) by the same procedure (with adjuvant) described in the previous study (Klecak and Ciullo, 1984a). Photosensitisation to AHTN for 10/10 animals was confirmed. These 10 photosensitised guinea pigs were then rechallenged with 1.0, 0.3, 0.1 or 0.03% ethanolic solutions of AHTN and each of its 4 photodegradation products with no UV-A irradiation in order to detect the degradation products responsible for the photosensitisation effect. Positive skin reactions were seen in 10/10 animals with 2 of the photodegradation products. In a separate experiment, another group of 10 guinea pigs was tested for photosensitisation with a 5% ethanolic solution of AHTN in the presence of Parsol 1789 (a UV-A filter) and the rate of photosensitisation was reportedly decreased to 6/10 animals (no details presented). When these 10 sensitised animals were rechallenged with the photodegradation products, one caused sensitisation reactions in 6/10 and another in 2/10 (again detail not given) (Klecak and Ciullo, 1985).

In a GLP compliant study, the nuchal area of 5 female and 4 male albino guinea pigs was shaved and injected with 4 doses of 0.1 ml of FCA followed by application of 0.1 ml/8 cm^2 of a 10% solution of AHTN (Fixolide – purity >98%) which in turn was followed by irradiation with UVB (≥1.8J) and UVA (≥ 20 J UVA). This dosing, but without the FCA, was repeated for a total of 5 applications plus irradiation in 2 weeks. Twelve days later, challenge was with 1, 0.3 or 0.1% solutions (0.025 ml/2 cm^2) on each of the flanks followed by 20 J UVA/cm^2 on 1 flank only. Reactions were read at 24 and 48 hr after challenge. There were 9/9 reactions at 1%; 8/9 at 0.3 % and 1/9 at 0.1 % with irradiation but no reactions without (Klecak, 1990).

In a GLP compliant photosensitization assay, 3 groups of 10 female Hartley guinea pigs were treated five consecutive days each week for 3 weeks on shaved and slightly abraded (every other day) skin with 100 µL (area approximately 1 cm^2) of 1% AHTN (1:1 mixture of "Tonalid and Fixolide – purity >98%) in absolute ethanol followed approximately 20-30 min

after application by irradiation with broad–band UVA (7 W/cm^2) and some UV–B light (25-35 μW/cm^2 at 2 cm) (group designation: AHTN + irradiation). The site of application was the left dorsal area. The 1% induction concentration was chosen based on the observation (see above) that this dose produced mild photoirritation. In addition, one group of 10 animals was treated with 1% AHTN in a similar way, but not irradiated (AHTN-irr; to detect non-photosensitisation), and 4 groups were treated with vehicle + irradiation (VH+irr) but not with AHTN. Two weeks after the induction phase, the animals in the four VH+irr groups were treated 5 times at 1 hr intervals with 100 μl of 0, 0.3%, 0.6% or 1% AHTN solutions followed by UV irradiation at 20-30 min after the last challenge dose. Similarly, the three AHTN+irr groups received 0.3, 0.6 and 1% AHTN challenges with irradiation and the AHTN-irr received a 1% AHTN challenge without irradiation. 6-Methylcoumarin (6-MC; induction 1%, challenge 0.3%), a known photosensitiser, was administered to 5 guinea pigs in the same manner to serve as a positive control. Challenge sites were then scored for erythema and oedema 24 and 48 hrs after the challenge application by visual evaluation and by measuring the size of the erythematous areas.

In the uninduced animals (VH+irr), the challenges with 0.3, 0.6 or 1% AHTN followed by irradiation caused a challenge dose-related response of photoirritancy after visual evaluation. A more pronounced response was observed in the induced (AHTN+irr) animals after the challenge but the magnitude of this response was similar in all challenge groups. After the 1% AHTN challenge, the photoirritation response (in the VH+irr group) and the photo-sensitisation response (in the AHTN+irr group) were essentially the same. The differences between the VH+irr and AHTN+irr groups were maintained for up to two days after challenge end of the observation period). No hypersensitivity was observed in the AHTN-irr group.

In contrast to the visual scoring, a clear challenge dose-related response was observed in the size of the erythematous area in the AHTN+irr induced animals, and in any case this response was larger than that observed in the VH+ir animals which were challenged with 1% AHTN+irr. The magnitude of the visual scoring with 6-MC was about similar to that of of the 1% AHTN challenge group, but the size of the erythematous area with any challenge of AHTN was larger than that with 6-MC.

Induced and uninduced animals were held for rechallenge with 0.3% AHTN at 5 and 18 wks after the initial challenge. A clear photosensitising response was obtained with mean scores essentially the same as at the original challenge. In this rechallenge study the challenge dose was applied either dissolved in ethanol or dissolved in dimethyl phthalate (DMP). With DMP a much less severe response was obtained than with ethanol. In addition with DMP no signs of photoirritation were observed in the uninduced animals, while with 0.3% in ethanol an indication of photoirritation was obtained (Stern, 1994).

In a GLP compliant photosensitization assay 0.2 ml of a 2% solution of AHTN (Tonalid ref. 18188 – purity >98%) in ethanol was applied once daily for 5 days to the shaved dorsal neck region (area 6 cm^2) of 10 male guinea pigs (Cpb:GpHi 65) followed by irradiation for 30 min using a Philips UV TL fluorescent lamp (40w/08, F40 Tl2 BLB; 310-420 nm distance to lamp 37 cm). At the mid-dorsal region of the back an area was treated with solvent only. During the induction phase slight erythema was observed at the AHTN-treated sites at days 3-5 after the irradiation in 2 to 4 animals. Ten days after the last topical induction a 2% solution was again applied to the shaved skin of induced and control animals followed by exposure to UV irradiation in the same manner as the induction doses. Skin readings were made after 24, 48 and 72 hr. No significant reactions were seen with the test solutions or with the solvent control (5 animals only) after the challenge at any time point (Prinsen & Til, 1985a).

The above test (also GLP) was repeated exactly with a second sample of AHTN (Tonalid ref 17855 – purity >98%) and in one or two animals very slight erythema on the AHTN-treated sites was seen at days 3 to 5 of the induction phase. Again, no indications for photosensitisation were obtained after the challenge at any time point (Prinsen & Til, 1985b).

A non-GLP repeated insult patch test with irradiation (photo-RIPT) with 1% and 5% AHTN (Fixolide/Tonalid 1:1) each in ethanol and dimethyl phthalate (4 separate dosing solutions) was carried out on each inner forearm (one for irradiation and one for control) of 25 human subjects with and without a non–erythemogenic UV–A radiation of 10–15 J/cm^2 (320-400 nm; peak at 365 nm). On the site to be irradiated, 0.01 ml of test material was applied directly to the site followed by irradiation for 45 min to 1 hr. These sites and the non-irradiated sites were then covered with Parke-Davis Readi-Bandage occlusive patches to which had been applied approximately 0.2 ml additional test material and the patch left in place for 24 hr. This was repeated 3 times per week for 3 weeks. Patched sites were scored 48 hr after application. After 14 days rest, challenge application were made in the same manner as the induction applications but on previously unpatched sites. The sites were scored 24 and 48 hr after application/irradiation. Barely perceptible to mild transient responses where seen throughout the induction with the 5% ethanol solutions in 5/25 persons, sometimes on irradiated and sometimes on non-irradiated sites (scores are given for the 5% solution applications only). On challenge, 2 subjects, 12 and 24 showed similar reactions at 24 hr with irradiation but not without and no reactions at 48 hr. Two subjects, 7 and 21, showed similar reactions on both irradiated and non-irradiated sites at 24 but not 48 hr. One subject exhibited a minimal barely perceptible reaction with papular eruption at 48 hr but not at 24 hr and only with irradiation. This subject also had similar reactions with and without irradiation following the first induction patch. Because of the marginal nature of these reactions, follow-up tests were undertaken. Subject 20 was rechallenged with 5% in ethanol as above 2 weeks later and exhibited moderate erythema plus mild oedema with irradiation at 24 and 48 hr but also had a moderate erythema without oedema at 24 hr without irradiation, however in this case the reaction subsided to mild erythema at 48 hr. The subject was again rechallenged approximately 2 months later as above but this time with a 1% solution in ethanol applied on both a naïve site and a previously “reactive” site. With irradiation, a moderate level reaction was seen at the reactive site and a mild reaction at the naïve site at 24 hr with both subsiding to barely perceptible reactions at 48 hr. A barely perceptible reaction was also seen with solvent control both at 24 and 48 hr. Without irradiation, a mild reaction was seen on both the reactive site and the naïve site at 24 hr with both subsiding to barely perceptible at 48 hr. The solvent gave a barely perceptible reaction at 24 hr that disappeared by 48 hr. Subject 20, along with subjects 12 and 21 (subject 24 was no longer available) were again challenged approximately 3 months later with 1 and 5% solutions AHTN, ethanol solvent and dimethyl phthalate all without irradiation to determine skin reactivities to these materials. No reactions were seen on any subject. A final follow-up occurred with subject 20 about 1 month later (approximately 7 months from the original test conclusion). This time the challenge was as in the original test with 1 and 5% solutions in ethanol and in dimethyl phthalate (6 applications including the 2 solvent controls) with and without irradiation. This time there were no reactions with any application with or without irradiation at any time point. Based on the original marginal results and the extensive follow-ups, the authors concluded that there was no significant evidence of photo sensitisation with AHTN in ethanol or dimethyl phthalate at concentrations up to 5% (Frentzko & Shanahan, 1989).

Two human allergenicity and photoallergenicity studies using a modified repeated insult patch test (photo-RIPT) according to the procedure of Kaidbey and Kligman (1980) were conducted in accordance with applicable Good Clinical Practice guidelines. These two

tests were with 0.2 ml of a 10% solution of AHTN (purity not stated) in ethanol/DEP (75/25) applied over an area of 4 cm^2 under occlusive patches (Webril covered by an occlusive hypoallergenic tape) on 29 humans with two different methods of irradiation using a 1000 watt Xenon Arc Solar Simulator with UV-A/UV-B filters. Prior to induction, the minimal erythema dose (MED) for the radiation to be used was determined for each subject. Test solution was applied to 2 sites (one for irradiation and one for control) 2 times a week for 3 successive weeks. At 24 hrs after application of the induction dose, patches were removed and half of the sites were exposed to UVA/UVB (2 × MED of UVB with 5% UVA)(time period not given). After a 2-week rest period, challenge doses were applied in triplicate in the same manner as the induction doses and the sites scored at 1, 24, 48 and 72 hr after patch removal. One application site was not irradiated to monitor contact sensitisation, one site was irradiated after patch removal at 24 hr post application and one site was treated with AHTN and irradiated at 10 minutes after application. This latter site was not further covered with an occlusive patch. The irradiation consisted of 16-20 J/cm^2 of UV-A and 0.75 × MED of UV-B. During the challenges, at least one additional test article (2 photodegradation products of AHTN: "product 4001"; 0.1% and "product 4002"; 1%, and a 3% solution of AHTN in 75/25 ethanol/DEP irradiated sample with 10 J UV-A) was included in the test, and these were not irradiated after the application.

Slight to mild signs of dermal irritation were obtained during the induction period. However, these reactions were observed in both AHTN-treated and vehicle-treated sites at about the same rate. There was no clear increase in the severity of the dermal irritation during the progress of the induction phase. After the challenge, slight irritation was observed in irradiated sites more often than in non-irradiated sites. However, upon comparison differences between AHTN-treated and vehicle-control sites were not observed, neither with respect to severity nor with respect to incidence. No cross-reactions to the photodegradation products of AHTN were seen, either. (Mills, 1997).

Conclusion

No evidence of sensitisation potential for humans has been seen in three studies (RIPT and maximization tests).

The available data with guinea pigs provide equivocal evidence of weak sensitising potential in one animal study (limited reporting) but not in another.

AHTN can act as a photosensitiser in guinea pigs at 1% although the results from these tests are difficult to interpret because AHTN is photoirritant to guinea pigs and there was no clear dose response on challenge. There is some evidence that the reactions seen with guinea pigs are the result of photodegradation of AHTN.

Tests on humans reveal no significant evidence of photosensitisation at levels up to 10%. Based on all data available AHTN is not considered to be photosensitiser as used in household products.

5.2.1.5 Repeated Dose Toxicity

Oral studies

A GLP compliant 28-day oral study with HanIbm:Wistar (SPF) rats according to OECD Guideline No. 407 was conducted by gavage dosing of groups five males (weights 175-195 g) and five females (weights 150-167 g) with 0 (control), 1, 3 or 10 mg/kg bw/day AHTN (Fixolide; purity 98.9%) in Oleum maydis germinis (total dose volume 10 ml/kg bw). There

were no mortalities, compound-related incompatibility reactions, ophthalmological changes, food consumption changes, body weight changes, effects on haematological or clinical chemical parameters, necropsy or histopathology findings (Dotti et al., 1993). There were no adverse effects at the highest dose tested, 10 mg/kg bw/day.

As described in a 13-week dietary toxicity study (see study by Lambert and Hopkins (1996) below), the doses to be used in this 13-week study were determined in a 2-week oral (dietary) range finding and palatability study. In the 2-week study, groups of 5 male and female Crl:CD(SD)BR rats were administered AHTN (purity not given in report but confirmed to be >98%) by dietary admixture at achieved doses of 0, 33, 88 and 169 mg/kg bw/day for males and 0, 32, 91 and 150 mg/kg bw/day for females. All animals in the high dose group were sacrificed prematurely on day 5 due to marked reduction in food consumption and bodyweight losses. Food consumption and body weight losses were slightly reduced at the mid-dose. A slightly higher absolute liver weight was noted in the females at the 2 lower doses and relative liver/bodyweights and kidney/bodyweights were increased in males and females at the mid and high dose. Relative liver weight was also increased in females at the low dose. Upon histopathology, hepatocyte fine vacuolation was observed in all groups, including controls, but with increased severity in mid dose males and high dose males and females. Based on these findings, the high dose for the 90-day study was selected to be 50 mg/kg bw/day.

In a GLP compliant study according to OECD Guideline 408, 5 groups of 15 male (weight 224-301 g) and 15 female (weight 157-214 g) Crl:CD(SD)BR rats received by dietary admixture nominal doses of 0 (control), 1.5, 5, 15 and 50 mg AHTN/kg bw/day for 13 weeks. AHTN (purity 99.3%). Analyses of diet indicated that desired homogeneity was reached. The concentrations of AHTN in the test diets were adjusted weekly. The mean achieved daily intakes of AHTN were 1.6, 5.0, 15.2 and 50.9 mg/kg bw for males and 1.5, 5.1, 15.1 and 50.8 mg/kg bw for females. After the treatment period, 3 females and 3 males 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 (at week 13 and at the end of the treatment-free period, only controls and high dose animals), urinalysis (at weeks 6 and 12 of treatment and at the end of the treatment-free period), haematology and clinical chemistry (at weeks 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 during the study. No clinical signs or ophthalmological abnormalities attributable to the administration of AHTN were noted during the study. During treatment, the mean body weight gain of males and females given 50 mg/kg bw/day was statistically significantly lower than that of controls (78 and 88% of controls, respectively). This improved upon cessation of treatment. Treatment with AHTN did not affect food consumption.

A reduction in red cell count, haemoglobin concentration and packed cell volume were observed at weeks 7 and 13 for males and females at 50 mg/kg bw/day (see Table 4). At 7 weeks but not at 13 weeks the animals at that dose showed polychromasia and anisocytosis. These findings were also observed in some animals of the two mid dose groups. A small but statistically significant prolonged prothrombin time was noted at week 13 for males and females given 5, 15 and 50 mg/kg bw/day but the values within the range of historical controls (except for the high dose males), and not clearly related to dose. Males at

50 mg/kg bw/day had statistically significantly higher white blood cell counts (WBC) at weeks 7 and 13. After the treatment-free period, no statistically significant differences were observed, but females and males at the high dose group still had slightly prolonged prothrombin time and higher WBC, respectively.

At weeks 7 and 13, in the male high dose group higher alkaline phosphatase and alanine aminotransferase activities were found. A higher A/G ratio was noted at week 13 for males and females given 15 mg/kg bw/day and at weeks 7 and 13 for males and females given 50 mg/kg bw/day, in males related to a small but statistically significant reduction in total protein at the two highest doses after 7 weeks and at all doses after 13 weeks No significant differences were seen for these parameters after the treatment-free period.

A lower plasma glucose concentration was seen at week 7 in males given 15 and 50 mg/kg bw/day, and in males of all dose groups at week 13. A reduction in plasma cholesterol was observed at weeks 7 and 13 for males and females given 15 and 50 mg/kg bw/day. Plasma triglyceride concentration was reduced in all dose groups in week 7 and at 5, 15 and 50 mg/kg bw/day in week 13. However, at least in the two highest dose groups, a clear dose response was seen. No values were significantly different from controls after the treatment-free period.

Table 4 – Selected hematological parameters for AHTN

Dose	PT M	PT F	Chol	Chol	Chol	Chol	Trigs	Trigs	Trigs	Trigs
	W13	W13	M 7		M 13	F 13	M 7	F 7	M 13	F 13
0	15.3	16.4	69	79	69	69	66	53	88	57
1.5	15.6	16.7	69	70	68	63	61	46*	86	54
5	15.9*	17.0*	64	73	62	62	54*	42*	69*	48*
15	15.9*	16.9*	57*	69*	53*	59*	48*	41*	59*	48*
50	16.7*	17.0*	44*	57*	41*	49*	37*	40*	41*	41*
HH	17.1	18.1	112	105	99	101	118	104	118	94
HM	15.2	16.2	76	75	66	73	78	57	85	54
HL	13.3	14.3	41	46	32	46	38	10	52	14

	Gluc	Gluc	Gluc	Gluc	A/G	A/G	A/G	A/G	Tprot	Tprot	Tprot	Tprot
	M 7	F 7	M 13	F 13	M 7	F 7	M 13	F 13	M 7	F 7	M 13	F 13
0	113	105	137	113	1.3	1.4	1.2	1.3	7.1	7.0	7.2	7.1
1.5	112	111	128*	120	1.4	1.4	1.3	1.3	6.9	6.9	7.0*	7.2
5	120	109	125*	110	1.3	1.4	1.3	1.2	6.9	6.8	7.0*	7.2
15	102*	98	119*	114	1.3	1.4	1.3*	1.4*	6.9*	7.0	6.9*	7.2
50	100*	100	113*	107	1.5*	1.6*	1.6*	1.4*	6.8*	6.7	6.8*	7.1
HH	127	127	136	137	1.5	1.6	1.3	1.3	7.4	7.2	7.9	8.4
HM	97	102	111	111	1.2	1.3	1.1	1.1	6.6	6.5	7.1	7.5
HL	67	78	85	85	0.9	1.0	0.9	0.9	5.8	5.8	6.3	6.6

	RBC	RBC	Hb M	Hb F	PCV	PCV	PCV	PCV	MCHC	MCHC	MCHC	MCHC
	M 13	F 13	13	W 13	M 7	F 7	M 13	F 13	M 7	F 7	M 13	F 13
0	9.1	8.5	16.9	16.5	45.1	44.5	46.9	45.7	36.5	35.5	36.0	36.2

1.5	8.9	8.8	16.6	16.8	44.7	44.2*	45.3	46.2	37.0	36.6*	36.7	36.4
5	9.1	8.6	16.7	16.6	45.2	42.7*	46.1	45.9	36.4	36.8*	36.2	36.2
15	9.0	8.2*	16.6	16.3	44.7	43.1*	46.1	44.3*	36.3	36.2*	35.9	36.7*
50	8.3*	8.0*	15.1*	15.5*	41.6*	41.8*	41.3*	42.4*	36.4	36.4*	36.6	36.6*
HH	9.8	9.2	17.6	17.4	49.7	48.1	50.7	49.3	37.5	37.9	37.4	37.5
HM	9.0	8.4	16.4	16.0	44.5	43.7	46.9	45.2	35.1	35.6	35.2	35.3
HL:	8.2	7.6	15.2	14.6	39.3	39.3	43.1	41.1	32.7	33.3	33.0	33.1

* Statistically significant, P<0/05 HH, HM, HL – Historical highs, means and lows

PT: Prothrombin time (sec)
Tprot: Total protein (g%)
Chol: Cholesterol (mg%)
RBC: Red blood cells count (10^6/µl)
Trigs: Triglycerides (mg%)
Hb: Haemaglobin concentration (g%)
Gluc: Glucose (mg%)
PCV: Packed cell volume (%)
A/G: Albumin/globulin ratio
MCHC: Mean cell haemoglobin concentration (g%)

A brown colouration of the urine was observed at weeks 6 and 12 for 5/15 males given 50 mg/kg bw/day (not seen after the treatment-free period). There were no effects on urine composition or cellularity.

The only organ weight changes attributable to AHTN were the increased absolute and relative liver weights in males and females given 50 mg/kg bw/day and in females only at 15 mg/kgbw/day (statistical significance was only attained for the relative weights at the highest dose). The liver changes were not seen after the treatment-free period and there was no increase in liver weight relative to brain weight. There were no histopathological changes in the liver. This indicates that the difference in liver/body weight could be due to the decreased body weight gains rather than an effect on the liver.

Upon macroscopy, abnormal green to dark coloured livers were observed in 11/12 males and 4/12 females given 50 mg/kg bw/day. Similar findings were noted in the mesenteric lymph nodes of 10/12 males and 3/12 females in the same group. No such discolouration was seen in the lower dose groups. A green colouration was seen in the lachrymal glands of females only at 50 mg/kg bw/day (8/12), 15 mg/kg bw/day (4/12), and 5 mg/kg bw/day (1/12). There were no associated histopathological findings in these organs. In addition, in liver there was no porphyrin accumulation. At the end of the treatment-free period only 1/3 males in the high dose group showed green coloured mesenteric lymph nodes, and 2/3 females in the same dose group showed green coloured lachrymal glands. The lachrymal gland colouration did not correlate with the colouration of the lymph nodes and livers (which were highly correlated with each other). Another finding upon macroscopy was uterine distension in females from all groups, including controls, but with slightly increased incidence/severity in treated females (dose-related). This finding was also observed microscopically, but it probably reflected normal cyclic change, given the absence of any other reproductive organ effect (males and females). At 5mg/kg bw/day there was evidence of colouring of the lachrymal glands in females only. However there were no histological differences observed in the lachrymal glands. Thus these findings are not considered toxicologically significant. There were some statistically significant differences in some biochemical and haematological values at this dose level. However, these were within historical control ranges and are not considered to be biologically significant. No adverse histopathological findings were found in the reproductive organs at any dose. Thus 5mg/kg bw/day is selected as the NOAEL (Lambert and Hopkins, 1996; Ford, 1998).

Dermal studies

Two studies with AHTN are poorly reported in Gressel, et al. 1980. One study involved dosing at 0, 1, 10 or 100 mg/kg/day for 13 weeks in groups of 15 female rats and the other was with 0, 9, 18 or 36 mg/kg/day in groups of 20 female rats for 26 weeks. The doses in ethanol (concentrations not reported) were applied unoccluded topically by gentle inunction to the anterior dorsal shaven skin (area of application not specified). No efforts were made to prevent oral ingestion. These studies were designed primarily to screen for possible neurotoxicity and acetyl ethyl tetramethyl tetralin (AETT), a known rat neurotoxin, was included as a positive control. These studies were not conducted in accordance with OECD guidelines and there is no evidence they were conducted in accordance with GLP.

A significant depression in body weight gain was seen in the 100 mg/kg/day, 13-week group and "a similar effect" was reported at interim and terminal sacrifice in the 26-week study at 36 mg/kg/day. A significant depression in haemoglobin, haematocrit and red blood cell count was reported for animals given 100 mg/kg/day in the 13-week study and a depressed haemoglobin level and red blood cell count at 36 mg/kg/day in the 26-week study. Elevated serum alkaline phosphatase was also seen at 100 mg/kg/day. Liver discoloration and prominent liver lobular patterns were observed in animals treated with 100 mg/kg/day and "similar effects" were reported at 36 mg/kg/day after 26 weeks. Relative and absolute liver weight increases were seen at 100 mg/kg/day. After 26 weeks, animals treated with AHTN at 36 and 18 mg/kg/day also demonstrated elevated liver weights but it is not stated whether these were relative, absolute or even statistically significant. In the 100 mg/kg/day, 13-week group moderate degrees of hepatocytomegaly and minimal to moderate deposition of an iron positive pigment were observed and "a similar, but less pronounced effect" was reported for the 36 mg/kg/day group. For none of these effects is any quantitative data given; thus, it is impossible to determine the severity of the effects or any dose response.

However, given the consistency of effects at 100 mg/kg/day at 13 weeks and 36 mg/kg/day at 26 weeks, along with what appears to be a dose response, these treatments must be considered to cause adverse effects. The problem is that the studies were limitedly reported and the severity of these effects cannot be judged. Additionally, no caution was taken to prevent oral intake so the exact dose cannot be determined. The most serious omission is data on the increased liver weight at 18 mg/kg for 26 weeks. If this is a mild adaptive effect not associated with any histopathology as it appears, then it could be argued that 18 mg/kg is the NOAEL. There is still the problem, however, of not being able to accurately determine the dose.

Clear evidence of neurotoxicity, both clinically and pathologically, was seen with the positive control AETT, but no such evidence for AHTN was seen in either study at any dose level

Because of 1) the uncertainties in the severity of the effects reported, 2) the study was conducted without collar or occlusion to prevent oral intake of compound making it impossible to determine actual exposures and 3) the area of application was not reported, this study should not be used to determine a dermal NOAEL (Gressel et al., 1980; Ford, 1998).

In a14-week dermal study of another fragrance material (identity deleted), AHTN (10% in ethanol) was included "for comparison" at a dose initially of 100 mg/kg/day to female Wistar rats. The alcoholic AHTN solution was rubbed into the shaved skin daily with no collars or occlusion to prevent oral intake. This produced such strong irritation within a few days of initiation, that growth was completely stunted. Because of this, the dose was reduced to 10 mg/kg/day on day 8 and onwards. The skin gradually recovered and weight gain became normal one week after the lowering of the dose, however, "erythema and inflammations"

were still seen at the end of the study. After 14 weeks roughness and scaliness of the treated skin-area was seen in 1/10 animals at the dose level (reversible). Slight to moderate skin lesions, viz. hyperkeratosis, parakeratosis and acanthosis, occurred too. 3/10 animals showed dark-colouration of kidneys (reversible). The reported growth inhibition and other effects can be attributed to the initial severe irritation (Til & Kuiper, 1978).

Because, 1) the effects reported appear to be a consequence of the dermal irritation, 2) the dose was changed during the conduct of the study, 3) there was no NOAEL, and 4) because the methodology was conducted without collar or occlusion to prevent oral intake, this study should not be used to determine an NOAEL

Inhalation

No data available.

Intraperitoneal

In order to study whether AHTN had any phenobarbital (PB)- and/or 3-methylcholanthrene (3MC)-like inducing properties, groups of 4 male Sprague-Dawley rats (weight 130-150 g) were treated i.p. with 0, 15 or 100 mg/kg bw/day AHTN (purity >99%) in DMSO for 3 consecutive days. At 24 hrs after the last injection liver microsomes were prepared from these animals and the activities of 7-ethoxyresorufin-O-deethylase (inducible by 3MC) and 7-pentoxyresorufin-O-deethylase (inducible by PB) were measured. At both dose levels tested, AHTN did not enhance the activities of the two liver enzymes (100 mg/kg bw even resulted in a strong decrease in activities of both enzymes). There was also no induction when the animals were sacrificed 4, 7 or 10 days after the last injection (Steinberg et al., 1999).

The same authors also studied the peroxisome-proliferating potential of AHTN, using the same dosing regimen as described above, with sacrifice of the animals at 5 days after the last i.p. injection. Neither at 15 nor at 100 mg/kg bw/day did AHTN enhance hepatic peroxisomal palmitoyl-CoA β-oxidation activity (Steinberg et al., 1999).

Conclusion

In a 90-day study in rats, clear but mild haematological effects were seen at the highest dose administered, 50 mg/kg bw/day. These effects may be associated with observations of dark discolouration of the liver and mesenteric lymph nodes seen in most high dose animals but in no animals at lower doses. Observations in animals maintained on a treatment free regime for 28 days following the 90-day treatment period indicate that the effects are reversible. Although the differences from controls were small and generally within historical ranges seen for rats in this laboratory, the overall pattern is such it cannot be excluded that these effects are of adverse nature despite the author's conclusion that these effects "were considered to be insufficient to have compromised the animals and to be of minor toxicological significance."

Small differences from controls were seen in some of the same parameters at 15 mg/kg bw but all of these differences were within the range of historical controls.

A NOAEL of 5 mg/kg bw/day can be concluded based on a careful analysis of all available data and is used in risk characterization.

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

5.2.1.6 Genetic Toxicity

Table 5. Mutagenicity studies available for AHTN

Type	Activation	Doses	Results	GLP	OECD	Reference
in vitro Bacterial (*S. typhimurium and E. coli*) Reverse Mutation Assay	with and without S-9	8, 40, 200, 1000, 5000 µg/plate	negative	Yes	471	Gocke, 1993; Api & San, 1999
in vitro Bacterial (*S. typhimurium)* Reverse Mutation Assay	with and without S-9	5, 16.6, 50, 166.6, 500 ug/plate	negative	No	OECD-like	Mersch-Sunderman et al., 1998a
in vitro Cytogenetic Assay with Chinese Hamster ovary	with and without S-9	5.9, 11.7 or 23.4 µg/ml w/o S-9 and 17.8, 20, 25 µg/ml & 3.9, 7.8 and 15.6 µg/ ml w S-9	negative	Yes	473	Curry, 1995; Api & San, 1999
in vitro Sister Chromatid Exchange Assay with human lymphocytes	with and without S-9	2.5-40 µg/ml	negative	No	OECD-like	Steinberg et al., 1999
in vitro Sister Chromatid Exchange Assay with human lymphocytes	with and without S-9	00.05, 0.49, 4.85, 48.5, 97, 194 µM	negative	No	OECD-like	Kevekordes et al., 1998
in vitro Unscheduled DNA synthesis with rat hepatocytes		0.1, 0.97, 9.7, 97, 194 and 387 µM	negative	Yes	482	San & Sly, 1994; Api & San, 1999
in vitro Micronucleus Test (human lymphocyte cells)	with and without S-9	0.025, 0.25, 2.43, 24.25, 48.5, 97 µM	negative	No	other	Kevekordes et al., 1997
in vitro Micronucleus Test (human hepatoma cells)	with and without S-9	0.05, 0.49, 4.85, 48.5, 97, 194 µM	negative	No	other	Kevekordes et al., 1997
in vitro SOS Induction with *E. coli*	with and without S-9	0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50 ug/assay	negative	No	other	Mersch-Sunderman et al., 1998b
in vivo Mouse Micronucleus Assay	na	400, 800, or 1600 mg/kg	negative	Yes	474	Gudi & Ritter, 1997 ; Api & San, 1999

a) In vivo

In a micronucleus test according to OECD guideline 474, groups of 5 male (weight 28-37 g) and 5 female (weight 24.5-31 g) ICR mice were dosed with 400, 800, or 1600 mg/kg bw AHTN (in corn oil; purity not reported) by intraperitoneal injection at a constant volume of 20 ml/kg bw. The high dose was selected to be 80% of the estimated intraperitoneal LD_{50} (see section 4.1.2.2.1). 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 occurred. Clinical signs consisted of lethargy at all dose levels and diarrhoea at 1600 mg/kg bw. Moderate reductions (up to 23%) in the ratio of PCE to total erythrocytes were observed in some treated groups suggesting 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 AHTN-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. (Gudi and Ritter, 1997; Api and San, 1999).

b) In vitro

AHTN (Fixolide; purity >98%) in DMSO was tested in the Ames test according to OECD guideline 471 using seven strains (*Salmonella typhimurium* TA 1535, TA 1537, TA 97, TA 98, TA 100, TA 102 and *Escherichia coli* WP2 uvrA) and appropriate positive controls. Two versions of the Ames test were used, the standard plate incorporation method and the preincubation method. In both procedures the substance was tested in absence and presence of S9 mix (from phenobarbital/β-naphthoflavone treated rats) at doses of 8, 40, 200, 1000 and 5000 µg/plate. No significant cytotoxicity was seen at any dose but at the three highest doses signs of limit of solubility were seen (milky appearance). No significant increase in the number of revertant colonies was observed for any of the seven test strains with AHTN by either procedure. All positive controls gave acceptable responses (Gocke, 1993; Api and San, 1999).

An Ames test (standard plate incorporation assay) was conducted with AHTN (Tonalid; purity not reported) 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 ug/plate (limit of solubility). All positive controls significantly increased the number of revertants. No significant increase in revertants was seen with AHTN at any dose with or without activation (Mersch–Sundermann et al., 1998a).

A cytogenetics assay with Chinese Hamster ovary (CHO-K_1) cells was conducted with AHTN (in acetone; purity not reported) according to OECD Guideline 473 at concentrations of 5.9, 11.7 or 23.4 µg/ml in the non-activated study, using 4, 20 and 44 hr exposure periods. In the S-9 (from rat liver induced by Aroclor 1254) activated study, dose levels of 17.8, 20 and 25 µg/ml were tested for the 4-hr exposure period with a 20-hr harvest time and at dose levels of 3.9, 7.8 and 15.6 µg/ml for the 4-hr exposure period with a 44-hr harvest time. At the 4, 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. 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. The mitotic index was significantly lowered at the highest dose in all cases. Positive controls caused increases in structural (significant) and numerical aberrations (significant in the case of B(a)P) in all cases. No significant increases in structural or numerical chromosome aberrations were observed for AHTN without metabolic activation at any dose. With metabolic activation, AHTN induced statistically significant increases in structural aberrations at all doses at the 20-hr harvest time (not dose-related and with significant cytotoxicity at all doses), as well as a statistically significant increase in numerical aberrations at the highest dose at the 44-hr harvest time. The latter increase was, however, still within historical control range. There was no dose response observed in this portion of the assay. Hence, it is concluded AHTN to be negative for structural and numerical chromosome aberrations in this test (Curry, 1995; Api and San, 1999).

Steinberg et al. (1999) studied the ability of AHTN (purity 99%) to induce sister-chromatid exchanges (SCEs) in cultured human lymphocytes obtained from healthy non-smoking donors ranging in age from 25-30 years. Cultures were treated for 24 hr with concentrations of 2.5-40 µg/ml AHTN in DMSO with or without rat liver S9 (Aroclor 1254-induced) metabolic activation. The method used resembled OECD guideline 479. After harvest, the cells were

scored for sister-chromatid exchanges. Cyclophosphamide (60 µg/ml) and ethyl methanesulfonate (120 µg/ml) were used as positive controls. Whereas the positive controls produced significant increases in sister-chromatid exchanges with (cyclophosphamide) and without (ethyl methanesulfonate) metabolic activation, AHTN up to 20 µg/ml caused no significant induction of sister-chromatid exchanges. Cytotoxicity was observed at 40 µg/ml AHTN.

The ability of AHTN (Tonalid; purity >98%)) to induce SCEs was evaluated using cultured human lymphocytes obtained from healthy non-smoking donors ranging in age from 25-35 years. Cultures were treated for 24 hr with concentrations of 0.025, 0.25, 2.43, 24.25, 48.5 or 97 µM AHTN (in DMSO) with or without rat liver S9 (Aroclor 1254-induced) metabolic activation. The method used resembled OECD guideline 479. After harvest, the cells were scored for sister-chromatid exchanges. Cyclophosphamide (used as positive control) produced a significant increase in sister-chromatid exchanges. Concentrations of AHTN up to 48.5 µM caused no significant induction of sister-chromatid exchanges (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 AHTN (purity not reported) in acetone in primary rat hepatocytes at concentrations of 0.15, 0.50, 1.5, 5.0 and 15 µg/ml (50-5000 µg/ml proved too toxic to be evaluated). 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 AHTN up to and including 15 µg/ml although this dose did induce significant cytotoxicity (San and Sly, 1994; Api and San, 1999).

An *in vitro* micronucleus test was conducted with AHTN (Tonalid; purity >98%) at concentrations of 0.05, 0.49, 4.85, 48.5, 97 or 194 µM using human peripheral lymphocyte cultures obtained from healthy non-smoking donors aged 25-35 years. After induction of mitosis, AHTN (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 AHTN 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 AHTN (Tonalid; purity >98%) at concentrations of 0.1, 0.97, 9.7, 97, 194 and 387 µM in DMSO using metabolically competent human hepatoma cells (Hep G2 line). After two hr incubation, the cells were harvested and scored for micronuclei in binucleated cells. The positive control (cyclophosphamide) significantly increased the frequency of micronuclei. No significant increase in the frequency of micronuclei was seen with AHTN at concentrations 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 *sfiA::lacZ* with AHTN (Tonalid; purity >98%) 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) ug/assay 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 the 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 AHTN at any dose (Mersch-Sundermann et al., 1998b).

Conclusion

AHTN 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. Hence, it is concluded that AHTN is a non-genotoxic substance.

5.2.1.7 Carcinogenicity

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

ATHN has been tested for liver tumour initiating and promoting activity in rats exposed to human-relevant doses. Female and male juvenile Wistar rats (5 weeks old at start) were exposed by intraperitoneal injections for 90 days to AHTN dissolved in isopropyl myristate either alone at 300 µg/kg bw/day or following a single i.p. dose of diethylnitrosamine (DEN) (100 mg/kg bw day) at 1, 10, 100 or 300 µg/kg bw/day. Thereafter the liver architecture as well as the presence of placental glutathione S-transferase (GST-P)-positive hepatic lesions was assessed. In male animals receiving AHTN alone or in combination with DEN the number of GST-P-positive single hepatocytes was similar to that of solvent-control treated rats, while GST-P-positive mini-foci and foci were not observed. In the case of female rats the number of GST-P-positive single hepatocytes and mini-foci in AHTN-treated rats was similar to that in untreated animals, whereas in those animals receiving AHTN either alone or in combination with DEN, GST-P-positive foci could not be detected or were present in a number as similar to that in untreated rats. In conclusion it has been shown that AHTN administered over a 90-day period in concentrations similar to those taken up daily by humans does not lead to an increase in GSTP positive foci in the liver. (Steinberg, et al., 2001).

Conclusion

AHTN is demonstrated to be not genotoxic. There are no indications from repeated dose toxicity studies of potential for carcinogenicity. Moreover, the chemical structure of AHTN does not have structural alerts for carcinogenicity (Ford, et al., 2000). It has been shown that AHTN has no liver tumour initiating and promoting activity in rats exposed to human-relevant doses. AHTN 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). 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 AHTN is a reproductive toxicant.

5.2.1.9 Developmental Toxicity/Teratogenicity

In a study designed to determine the effects of AHTN on the neonate when exposed through nursing, AHTN (purity 99.3%) was administered at dosages of 0, 2, 6 or 20 mg/kg bw/day 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 (litters were standardised to 8 pups on Day 4 post partum). 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 AHTN *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 the pharmacokinetic study in pregnant/lactating rats (Hawkins, et al., 1996).AHTN levels in mother's milk up to 1.9 and 25 mg AHTN equivalents (including also metabolite)/l were found at oral doses of 2 and 20 mg ^{14}C-AHTN/kg bw/ day, respectively, to the dams. 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. There were no adverse effects to the dams or offspring up to and including the highest dose level (20 mg/kg bw/day). 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 (Jones, et al., 1996;Ford and Bottomley, 1997).

A GLP compliant dosage-range finding study was conducted to provide information for the selection of dosages to be used in the developmental study. In this study, groups of 8 pregnant Sprague-Dawley rats were administered AHTN (purity not stated) in corn oil by gavage (5 ml/kg bw) at doses of 10, 25, 50 or 100 mg/kg bw on days 7 through 17 of pregnancy. The control group consisted of 19 animals and received corn oil only. One rat in the high dose group was sacrificed in moribund condition on day 19 and all other animals were sacrificed at day 20. All (including the one intercurrent death) were studied for gross pathology. Uteri were opened and foetal body weights and gross external alterations were recorded. Foetuses with anomalies were fixed in Bouin's solution. In the high dose group decreased motor activity, chromorhinorrhea, perioral substance, excessive salvation, dehydration, emaciation,

perivaginal substance alopecia and urine stained fur were observed. Eight of the animals in this group had green livers, 3 had small spleens and 2 had green amniotic sacs for all foetuses. In the other dose-groups no treatment related necropsy findings were recorded.

The animals in the 50 mg/kg group had decreased body weight gain and in the 100 mg/kg group weight loss was recorded. Reduced weight gains were also seen at 25 mg/kg bw during days 7-10 and 12-15. Feed intake was reduced at 50 and 100 mg/kg bw, but not in the other dose groups. No litter parameters or external alterations were seen at 50 mg/kg bw or lower. Three foetuses from two litters in the high dose group had whole body oedema. Based on these findings, doses of 5, 15 and 50 mg/kg bw were chosen for the main study (Christian et al., 1997a, 1999).

In a GLP compliant study, AHTN (purity not stated) 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, 5, 15 and 50 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.

In the 50 mg/kg bw/day dose group a statistically significant number of rats had localised alopecia and colour changes in the liver (green or mottled green and dark red). The 50 mg/kg bw/day group had an initial weight loss and a significant reduction in maternal bodyweight gains accompanied by a decreased food consumption.

The 5 mg/kg and the 15 mg/kg dose group animals had a transient reduced body weight gain during the first days of the dosing period, but at the end of the study maternal body weights in these two groups were approximately similar to those from the control animals. Feed intake was significantly reduced at 15 mg/kg bw. There were no significant differences seen on clinical examination or at necropsy between the two lower dose groups and controls. Because there were no other signs of adverse effects at the 15 mg/kg bw level, this level can be considered the maternal NOAEL in this study.

Average foetal body weights were about 5% lower in the low dose group, about 3.5% lower in the mid dose group and about 6.5% lower in the high dose groups. There was a Statistically significant reduction in body weight in the low and high dose groups but not in the mid-dose. The decreases in bodyweight s observed in all three groups were well within historical ranges and because of the marginal dose relationship they were not considered biologically relevant. No effects were observed on numbers of implantations, dead or live foetuses, resorptions or foetal sex ratios.

There were no irreversible skeletal changes and all gross external, soft tissue or skeletal malformations in the foetuses were incidental and considered unrelated to the test article. Retardations in ossification were observed in foetuses from all four groups. Only for the numbers of caudal (in all test groups) and sternal centres (in the two higher dose groups) of ossification statistically significant decreases were observed however the decreases were not dose dependent. These can be considered to be a) related to the slightly decreased foetal body weights, b) not specific and c) not of biological significance. Therefore in this study no evidence for developmental toxicity was obtained, despite maternal exposure up to toxic

levels and the developmental NOAEL is 50 mg/kg bw/day, the highest dose administered. (Christian et al., 1997b, 1999).

Conclusion

In an oral peri/postnatal toxicity study (exposure of the F_1-generation to AHTN was only *in utero* during the perinatal phase or through any transfer in the milk of the lactating dams), no toxicity was seen at any dose level in the dams or their F1 and F2 offspring. 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-AHTN/kg bw per day. Levels up to 1.89 and 25 mg AHTN equivalents (i.e. AHTN + metabolites)/l of whole milk were reported, for maternal oral doses of 2 and 20 mg/kg bw/d, respectively. Actual intakes cannot be determined because milk consumption during nursing was not measured however these levels can be compared to the average level of 0.59 ppb with a maximum of 9.8 ppb in human milk samples.

In an oral development study, there were signs of maternal toxicity only at 50 mg/kg bw the highest dose administered. Developmental toxicity was not seen at any dose level. Therefore, the NOAEL for maternal toxicity can be established at 15 mg/kg bw/day and the developmental NOAEL is ≥50 mg/kg bw/day, the highest dose administered.

5.2.1.10 Additional data

Endocrine interactions

In a non-GLP study, AHTN in ethanol was added to transiently ERα- or ERβ- transfected human embryonal kidney 293 cells for 24 hr. AHTN 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, AHTN (purity 99%) 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 7 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. AHTN showed a higher and statistically significant rate of proliferation relative to the hormone-free control (ratio 1.53). This increase was 1×10^{-5} that of 17β-estradiol. (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 10 or 50 mg AHTN/kg for 2 weeks. This resulted in mean daily intakes of about 2 or 6.5 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 (0.14 mg) on days 1, 5, 9 and 12 of the study. The estradiol treated mice had significantly increased uterine weights and decreased thymus weights, but no effect on liver weight. AHTN had no significant effects on either organ, but it did cause an increase in absolute liver weight. (Seinen, et al., 1999).

Conclusion

AHTN has been reported to have very weak estrogenic potency *in vitro* but in an *in vivo* study, no such effects were seen. AHTN is thus not considered to produce endocrine disruption *in vivo*.

Toxicokinetics

The protocols of all studies in this section, were not specifically designed according to OECD Method 417, however, the basic guidance principles of OECD Method 417 were used for the evaluation of the test results. The following studies did not have enough details; the analyses of human fat and milk samples (Eschke, et al., 1995b and Rimkus and Wolf, 1996) and the *in vitro* dermal absorption studies with excised rat skin (Ashcroft and Hotchkiss, 1996) and with minipig skin (Hoffmann-LaRoche, 1983a).

In vivo studies

All available studies in this section were evaluated for information on the absorption, distribution, excretion and metabolism of AHTN 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 AHTN 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 (under occlusion) with 4.5 mg/kg bw of ^{14}C-AHTN (uniformly labelled in the aromatic ring – radiochemical purity 98.9%) in 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 (200 µl of a solution containing 4.55 mg ^{14}C-AHTN/ml). The solvent was allowed to evaporate for an unreported time before the area was occluded with aluminium foil. (This experiment was conducted for the purpose of obtaining ethical approval for the human simulated exposure experiment (see below) and thus, skin exposure limited to 6 hr). 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 (aluminium 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 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, all tissues (including untreated and treated skin) as well as the remaining carcass were analysed for radioactivity. Urine was collected at 0-6, 6-12, 12-24, 24-48, 48-72, 72-96 and 96-120 hr, faeces and air (0 – 48 hr only) were collected every 24 hr until 120 hr.

A majority (mean of 69.6%) of the applied material remained on the surface of the skin at the time of washing - 6 hr. Only 9.3% of the applied dose was found in excreta and tissues at that time point. During the exposure for 6 hrs a significant skin reservoir of material (approximately 13%) was formed. Some of this material may be absorbed, since up to 120 hr trace levels in organs are still being present. Based on the amount of radiolabel excreted (see below in Excretion section) combined with what remained in the tissues and carcass, but excluding the amount remaining in the skin at the treatment site at 120 hr (1.5%; see

Distribution section below), approximately 18.8% of the applied dose had been absorbed in 120 hrs. Analysis of the dressings applied after surface dose removal indicated that about 2% of the 13% of the material in the skin reservoir was lost to the dressing by reverse diffusion and/or desquamation. Average recovery of radiolabel (6 – 120 hr) was 91.2% (Ford et al., 1999; Aikens, 1995).

Radiolabelled (position and radiochemical purity not reported) AHTN, dissolved in methylcarbitol at concentrations of 1, 3 and 10 %, was applied to an intact excised minipig skin (strain and source not reported) area of 5 cm^2 at a dose of 12 µl/ cm^2 (= 120, 360 or 1200 µg/ cm^2). The receptor fluid was physiological saline in which the solubility of AHTN is not reported. Unabsorbed material (92.3%, 89.1% and 86.2% at 10, 3 and 1%, respectively) from the skin surface was removed after 16 hr. The stratum corneum was then removed by successive tape stripping, and the stratum corneum and the remaining stripped skin were then analysed for radiolabel. Applying doses of 1, 3 and 10% the amounts of labelled material in the horny layer were 5.5, 5.3, 4.4 % and 8.2, 5.6, 3.3 % in stripped skin. Penetration into the receptor fluid was undetectable after 16 hr (Hoffmann-LaRoche, 1983a). This study was not conducted under GLP. Whilst demonstrating that AHTN is only absorbed to a small extent, the study does not provide quantitative data because the limited solubility of AHTN in the receptor fluid. The solubility of AHTN in physiological saline would be too low to allow penetration in the absence of skin metabolism.

Distribution: The GLP rat *in vivo* dermal absorption study, described above (Ford et al., 1999; Aikens, 1995), shows that plasma levels have peaked between 6 hr (time of removal of dose from surface) and 12 hr. This conclusion is based on the similarity of concentrations at both times and the theory of absorption kinetics. Analyses of tissue levels indicate that approximately 1.7% of the absorbed radiolabel was present at 120 hr; at that time, also approximately 1.5% still remained in a skin reservoir at the treatment site. The large majority of the absorbed radiolabel was found in the large and small intestines and their contents (Table 6). In the large intestine the highest level in was reached after 12 hr, thus at a later time than in the plasma, this behaviour is consistent with biliary excretion. Levels in other organs and fat essentially reflected the plasma levels peaking at 6-12 hr and declining after that. The levels measured in tissues and organs not given in Table 6 never exceeded 0.3 µg equivalents/g tissue. Average recovery of radiolabel (6 – 120 hrs) was 91.2%.

Table 6 Distribution of radioactivity in selected tissues during 0.5 to 120 hours after dermal application of 14C-AHTN to male rats at a dose of 4.5 mg/kg bw over an area of 9 cm2 (as µg equivalents/g of tissue)

Tissues	Time (hr after initial application)								
	0.5	1	3	6	12	24	48	72	120
LI + contents	0.014	0.053	0.35	2.28	6.70	3.36	2.48	0.91	0.26
SI + contents	0.041	0.36	2.45	4.49	4.13	2.02	1.36	0.67	0.24
stomach + contents	0.003	0.005	0.032	0.108	0.074	0.040	0.016	0.009	0.008
Liver	0.039	0.17	0.48	1.01	1.35	0.86	1.01	0.84	0.54
Fat	0.006	0.035	0.16	0.32	0.35	0.27	0.30	0.22	0.12
Plasma	0.009	0.044	0.13	0.21	0.22	0.12	0.10	0.077	0.037
Adrenal glands	0.067	0.19	0.25	0.28	0.15	0.071	0.085	0.053	0.036

Tissues	Time (hr after initial application)								
	0.5	1	3	6	12	24	48	72	120
Kidneys	0.026	0.12	0.25	0.28	0.16	0.11	0.11	0.095	0.072
Thyroid	nd	0.14	0.44	0.30	0.22	0.084	0.14	0.078	0.049
Untreated Skin									
non-pigmented	0.010	0.044	0.14	0.23	0.20	0.13	0.067	0.047	0.029
pigmented	0.007	0.04	0.09	0.14	0.25	0.11	0.11	0.081	0.063

LI = Large intestine SI = Small intestine

Excretion: In the *in vivo* dermal absorption study (GLP) in rats (6 hr application under occlusion) described above, after 120 hr, 17.1% of the applied dose had been excreted (primarily in the faeces – 14.5%, with the remainder in the urine (+ cage washing) – 2.6%, none in the expired air) with the majority excreted within 48 hr (10.1%). The rate of excretion reflects the rate of absorption from the skin with a half-life of approximately 2 days. No attempt was made to characterize possible metabolites. Average recovery of radiolabel (6 – 120 hrs) was 91.2% (Ford et al., 1999; Aikens, 1995)

Metabolism: No data available.

Oral

Absorption: In a GLP compliant study designed to explore the coloured organs seen in some studies (see Repeated Dose Toxicity, section 4.1.2.6 below), AHTN (purity 98.8%) was administered in corn oil to groups of 5 male or 5 female Sprague-Dawley (Crl:CD BR; ~ 7 weeks old, individually housed) in daily doses of 15 or 100 mg/kg bw for 14 days followed by 2 daily doses of 15 or 100 mg/kg bw radiolabelled AHTN (uniformly labelled in the aromatic ring – radiochemical purity 97%). A third group (one animal per sex) was treated orally with a single dose of vehicle (Mazola corn oil) for 16 days and served as the control. Urine was collected 0-8, 8-24 and 24-48 hr after the first radiolabel dose and faeces were collected daily. Cage rinse was collected daily for 2 days post first radioactive dose. Twenty-four hours after the 2nd radioactive dose, animals were sacrificed by CO_2. Blood, liver, kidneys, lachrymal glands, mesenteric lymph nodes and any other tissues showing abnormal coloration due to the chemical treatment were collected (Wu, 2002).

Distribution: In the Wu (2002) study, liver, kidney, mesenteric lymph nodes and lachrymal glands showed a dark green to almost black discolouration in the high dose group (100 mg/kg bw/day). In addition, these animals showed a discolouration of the urine. Only slight discolouration of the internal organs was seen in the low dose (15 mg/kg bw/day) animals.

Table 7 Distribution into livers, kidneys, carcasses and spleens (females only) of AHTN after oral dosing in the rat (as % dose)

Group	Liver	Kidney	Carcass	Spleen
Male low dose	6.35 ± 0.83	0.09 ± 0.01	20.21 ± 7.61	-
Female low dose	4.52 ± 0.71	0.11 ± 0.03	23.90 ± 10.96	0.02 ± 0.01
Male high dose	4.46 ± 0.61	0.10 ± 0.02	15.07 ± 6.64	-

Group	Liver	Kidney	Carcass	Spleen
Female high dose	3.71 ± 0.48	0.11 ± 0.03	14.97 ± 7.49	0.02 ± 0.0

The relative distributions are shown in Table 7. Animals in the high dose group either lost weight or had a slower growth rate. Some animals had alopecia starting around 4 – 5 days of treatment.

In a GLP study, to pregnant Charles River CD rats ^{14}C- AHTN (uniformly labelled in the aromatic ring – radiolabel purity 98.0%) was administered daily by gavage from day 14 of gestation up to 7 days post-parturition at 2.0 mg/kg bw in corn oil (see section 4.1.2.1.4 below) and the plasma and milk levels of AHTN measured. At the end at 4 and 24 hr after the last dose, a single rat was sacrificed for whole body autoradiography. Radioactivity levels in the foetus were essentially undetectable (Hawkins, et al., 1996a).

Excretion: After oral administration of 14 daily doses of unlabelled AHTN followed by 2 days of oral dosing of radiolabelled material to rats (see Wu, 2002 above), all samples were processed for radioanalysis but only those reported below were reported. Pooled urine, faeces and liver/kidney samples from both dose groups per sex and collection time were analysed for metabolite radioprofiles by HPLC radiochromatography. Radioactivity levels in samples were determined by liquid scintillation counting. The results are shown in Table 8. Within 48 hours 66-67% (low dose) vs. 74-76% (high dose) radiolabel was excreted. In the low dose, the majority was in the faeces while in the high dose approximately equal amounts were found in faeces and urine plus cage rinse.

Table 8 Excretion of AHTN from rat

Group ID	Sex	% Dose in Urine	% Dose in Cage Rinse	% Dose in Faeces	% Dose in Tissues	% Dose in Recovered
1 (15 mg/kg bw/day)	M	11.04	2.92	52.18	26.66	92.80
	F	14.16	7.97	44.76	28.55	95.44
2 (100 mg/kg bw/day)	M	35.06	6.27	35.00	19.63	95.95
	F	28.20	3.98	41.74	18.82	92.74

Metabolism: In the two-week oral study (Wu, 2002) discussed above, the metabolic profile of [^{14}C]AHTN in urine, faeces and liver samples was evaluated by HPLC radiochromatography which revealed the formation of numerous and complex metabolites however, none of these were characterised. No unchanged AHTN was detected in the urine or liver however; extensive levels of AHTN (not reported) were seen in the faeces (especially in females at the high dose), possibly as a result of lack of absorption. Similar complex metabolites were seen in the liver but these were also not characterised. In the liver 63-73% of metabolites were not readily extractable but irreversibly bound. Attempts to relate metabolites to discolouration of organs were unsuccessful.

Intravenous

Distribution: In a GLP study, a single intravenous dose of 2 mg/kg bw ^{14}C-AHTN (uniformly labelled in the aromatic ring – radiochemical purity 98.4%) in 0.4 mg/ml ethanol/Emulphor EL 620/ Isotonic saline (1:1:7) solution was administrated into the tail vein to groups of four female Sprague-Dawley CD rats (bodyweight range 216-233 g). The

animals were sacrificed after 5, 15, 30 minutes 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 every 24 hr from the four animals sacrificed at day 7 (air only up to 48 hrs). The mean recovery of total radioactivity in these four animals represented 93.9% of the dose administered: 88.9% in excreta + cage washings, 3.6% in carcass and 1.4% in liver.

Maximum concentrations of total radioactivity were observed in all tissues at 5 min (earliest time of measurement) except for the fat where the maximum was reached at 2 hr (Table 9). Between 48 hr and 16 days, total radioactivity in plasma and fat decreased with half-lives of 1.9 days and 2.5 days, respectively; after 16 days the concentrations declined more slowly. Levels of radioactivity declined between 7 and 28 days half-lives of 15.2 days in whole blood, at 5.4 days in the liver and 11.2 days in the kidneys (Hawkins et al., 1997).

Table 9 Concentrations of radioactivity in tissues after an intravenous dose of 14C-AHTN to rats at a dose level of 2 mg/kg bw (in µg equivalents/g tissue).

	Tissues				
Time	Plasma	Whole Blood	Liver	Kidney	Fat
5 min	2.55	1.47	9.64	4.64	0.912
15 min	1.88	1.11	6.13	3.29	1.95
30 min	1.55	0.934	4.78	2.62	3.22
1 hr	1.66	1.02	4.94	1.81	3.20
2 hr	1.63	1.05	4.68	1.32	5.13
4 hr	1.83	1.18	5.10	1.02	4.36
6 hr	1.81	1.15	4.74	1.08	3.52
12 hr	1.47	1.05	4.54	0.803	3.52
24 hr	0.823	0.681	2.22	0.532	2.89
2 days	0.532	0.531	1.94	0.385	1.75
7 days	0.0929	0.225	0.655	0.176	0.356
16 days	0.00327	0.133	0.132	0.0807	0.0382
28 days	0.00110	0.0857	0.0423	0.0470	0.0132

In a GLP study, a nominal dose of 0.1 mg/kg bw ^{14}C-AHTN (actual dose 0.091 mg/kg bw; uniformly labelled in the aromatic ring – radiochemical purity >97%) in ethanol/ Emulphor EL 620/isotonic saline (1:1:7) solution was administered by intravenous injection into the ear vein of one male domestic pig (*Sus scrofa* of Large White Hybrid strain – age 8-12 weeks, bodyweight 33 kg). 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, 40 minutes and 1, 2, 4, 8, 12, 24 hr and 2, 3, 5, 7, 14, 21, and 28 days. Biopsies of skin and underlying fat tissue were taken at day 9, 16 and 28. The pig was sacrificed at 28 days. The recovery of total radioactivity (assessed in excreta only) was 98.4% of the administered dose.

The maximum concentrations of total radioactivity in whole blood and plasma were observed at 10 minutes (earliest collection) (see Table 10). Total radioactivity decreased rapidly in

blood and plasma with apparent half-lives of about 0.9 hr during the first two hr. Thereafter the concentrations declined at a slower rate, with half–lives of 190 hr in plasma and 270 hr in whole–blood during 48–672 hr. There was no obvious accumulation of radioactivity in blood cells. In underlying fat of the skin, the maximal concentration (earliest collection) was at 9 days (7.57 ng equiv./g). After that, the fat concentration decreased and it was <5.6 ng equiv./g 16 days after injection and <0.8 ng equiv./g after 28 days. In skin, 1.08 ng equiv./g was found at 9 days and <0.8 ng equiv./g at 16 and 28 days. There was no marked accumulation of AHTN in skin or fat (Girkin, 1997).

Table 10 Concentrations of radioactivity in blood and plasma after an intravenous dose of 14C- AHTN to a pig at a dose level of 0.1 mg/kg bw (in ng equivalents/g).

Time (hr)	Whole blood	Plasma
10 min	70.7	108
20 min	64.1	100
40 min	48.9	74.0
1	33.9	52.8
2	17.5	25.9
4	9.8	13.7
8	8.5	11.6
12	7.4	9.6
24	6.1	7.3
48	5.0	6.1
72	4.5	5.3
120	4.1	4.4
168	3.3	3.4
336	1.1	1.6
504	1.4	1.2
672	1.0	0.6

Metabolism: The urine data collected at the two (rat and pig) intravenous studies (GLP) described above were analysed for metabolites. Extraction of pig urine (collected at 6-48 hrs) and rat urine (collected at 0-24 hrs) with solvent E (chloroform/methanol/water/formic acid, 75/25/3/3 by volume) revealed at least 13 metabolites in pigs and 16 in rats (Table 11).

Table 12 Proportion of urinary metabolites as a percentage of dose after intravenous injection in rats or a pig – solvent E

Rf value	Pig urine 6-48 hr		Rat urine 0-24 hr	
	Untreated	Enzyme treated	Untreated	Enzyme treated
0.06	0.81	0.29	0.31	0.19

Rf value	Pig urine 6-48 hr		Rat urine 0-24 hr	
	Untreated	Enzyme treated	Untreated	Enzyme treated
0.20	*	*	0.22	0.17
0.26	0.12	*	0.86	0.75
0.31	0.10	*	0.64	0.43
0.36	0.16	0.06	4.0	3.86
0.43	*	*	0.13	0.09
0.47	*	*	0.29	0.60
0.52	0.19	*	0.50	
0.62	0.87	0.89	0.29	0.43
0.65	5.0	5.79	1.25	1.34
0.69	6.22	5.45	2.47	2.42
0.74	7.46	8.7	1.32	1.28
0.79	13.84	13.67	0.67	0.89
0.84	33.85	33.98	0.40	0.78
0.91	7.13	5.31	0.21	0.61
0.97	1.41	1.30	0.18	0.23
others	0.38	2.10	0.38	0.02

*Not detected

Extraction with solvent H (chloroform/methanol/ammonia, 80/20/1 by volume) revealed at least 9 metabolites in pigs and 8 in rats (Table 13). None of the metabolites were characterized other than by retention times (R_f). Treatment with glucuronidase or aryl sulphatase had little effect on the proportions of the main metabolites in rat and pig urine, but solvent H had somewhat more conjugates than solvent E. The parent compound was extracted from urinary radioactivity with solvent A (100% chloroform), but neither pig nor rat urine contained unchanged AHTN (Girkin, 1998). In addition, it should be noted that the dose excreted in rat urine (21.5%), was considerably less than in the pig (86.2%), so the percentage of most rat urinary metabolites detected are much lower.

Table 13 Proportion of urinary metabolites as a percentage of dose after intravenous injection in rats or a pig – solvent H

Rf value	Pig urine 6-48 hr		Rat urine 0-24 hr	
	Untreated	Enzyme treated	Untreated	Enzyme treated
0.03	3.44	3.16	1.85	1.14
0.12	4.34	2.75	3.44	3.44
0.21	0.12	1.79	*	*
0.25	1.97	1.14	3.02	2.81
0.34	5.49	5.19	3.07	3.00

Rf value	Pig urine 6-48 hr		Rat urine 0-24 hr	
	Untreated	Enzyme treated	Untreated	Enzyme treated
0.50	*	0.27	-	-
0.60	-	-	1.03	0.93
0.62	6.05	4.06	0.46	0.49
0.69	8.24	9.04	0.17	1.11
0.75	16.63	15.35	0.34	
0.82	20.36	19.02	*	
0.88	6.47	7.84	*	0.20
0.92	*	1.23	*	*
0.99	*	1.89	*	*
Others	4.38	4.81	0.73	0.99

*Not detected

Excretion: In the rat intravenous study (GLP) described above (Hawkins et al., 1997) the main route of excretion was by faeces (67.1% of the administered dose) and excretion in the urine was 21.5% of the administered dose. Most excretion took place in the first 48-72 hrs. An absence of excretion of radioactivity to the air was noted.

In the pig intravenous study (GLP) described above (Girkin, 1997) the main route of excretion was urinary (86.2% of total dose after 14 days) and total radioactivity recovered in faeces after 14 days represented 12.2 % of the administered dose. The process of elimination was rapid and mainly occurred during the first 48-72 hrs.

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-AHTN (uniformly labelled in the aromatic ring – radiolabel purity >97%) was administered by gavage to pregnant Charles River CD rats (n=18/group – bodyweights *ca* 250-400 g – age 10-15 wks) at 2.0 or 20 mg/kg bw as a solution in corn oil, 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, which takes place mainly before day 14. The sponsor was aware that some organogenesis occurs after day 14. At 4, 8 and 24 hr after administration of oxytocin and on days 3 and 7 post-parturition, milk samples of about 0.5 ml and blood samples of about 4 ml were obtained from 3 dams per dose level and analysed for radiolabel. The highest mean levels of radiolabel were found in the 4 hr plasma samples, declining to about 35% of that level at 24 hr after dosing (see Table 14). Lower levels were consistently seen after 7 days as opposed to 3 days indicating no significant accumulation. Plasma levels were roughly proportional to dose with plasma levels at a dose of 20 mg/kg bw/day approximately 10 fold higher than those at 2 mg/kg bw/day.

Table 14 Analysis of total radioactivity in plasma after daily oral administration of 14C-AHTN in µg equivalents AHTN/ml plasma

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

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	3.13 ± 0.40	25.1 ± 3.5
	8	1.72 ± 0.29	24.3 ± 6.4
	24	1.10 ± 0.18	9.98 ± 1.6
Day 7	4	2.41 ± 0.46	21.0 ± 2.2
	8	2.20 ± 1.02	17.3 ± 1.9
	24	0.86 ± 0.10	6.53 ± 0.9

Levels of total residue found in the milk (Table 15) were also highest at 4 hr after dosing declining 5 to 10 fold by 24 hr. Again lower levels were generally seen after 7 days dosing as compared to those after 3 days dosing (except for 2 mg/kg at 4 and 8 hrs) also consistent with no significant accumulation. Additionally, the major residues in the milk were not associated with a peak, which apparently co-chromatographed with AHTN. About 66-85% and 57-81% of the radioactivity was associated with other materials (metabolites) at the low and high dose, respectively. (Hawkins and Ford, 1996; Kirkpatrick, 1996).

Table 15 Analysis of total radioactivity and unchanged AHTN in milk after daily oral administration of 14C-AHTN in mg equivalents AHTN/kg.d milk

Milk collection after parturition	Time after oral administration(hours)	2 mg/kg/day			20 mg/kg/day		
		Total Mean radiolabel	AHTN Mean	Ratio AHTN/total residue	Total Mean radiolabel	AHTN Mean	Ratio AHTN/total residue
Day 3	4	1.45 ± 0.43	0.35 ± 0.25	0.22 ± 0.10	25.0 ± 11.1	9.43 ± 2.57	0.41 ± 0.10
	8	0.66 ± 0.12	0.10 ± 0.02	0.15 ± 0.05	10.5 ± 3.35	2.14 ± 1.39	0.19 ± 0.10
	24	0.31 ± 0.07	na	-	2.89 ± 0.71	na	-
Day 7	4	1.89 ± 0.57	0.63 ± 0.20	0.34 ± 0.02	18.0 ± 1.1	7.73 ± 0.71	0.43 ± 0.02
	8	0.87 ± 0.21	0.14 ± 0.05	0.15 ± 0.03	8.76 ± 2.40	2.95 ± 1.65	0.32 ± 0.09
	24	0.21 ± 0.06	na	-	1.55 ± 0.30	na	-

na : not analysed due to low radioactivity levels

In vitro studies

Absorption: The *in vitro* absorption of ^{14}C-AHTN (place of labelling not given) 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/hr. Dose solutions of 0.1% and 0.5% in an ethanol/DEP (75:25) vehicle (15 and 78 μg/cm^2, respectively) were applied to occluded (Teflon caps) and non-occluded systems. Receptor fluid was collected every 2 h for up to 72 h. At the end of the experiment, the skin surface was washed and swabbed, after which the skin was digested in methanolic sodium hydroxide. Radioactivity in receptor fluid, skin washes and skin was determined by liquid scintillation spectrometry. AHTN was poorly absorbed through non-occluded skin after 24 hr (0.28% of applied dose). Occlusion enhanced AHTN absorption at 24 hours to 3.0%. Significant amounts of radioactivity were recovered from within the skin

(at 24 h, 55% in both unoccluded and occluded skin). Over 48 h, AHTN continued to be absorbed into the receptor fluid and the total absorption at 48 h was enhanced by occlusion. No data were presented on the 48-72 hr time-period. Total recovery of radioactivity was not presented but was stated to be generally >80% (Ashcroft and Hotchkiss, 1996). Based on the use of a non-physiologically receptor fluid, no report of testing the integrity of the skin (the data were taken from a poster presentation) and poor recovery of radiolabel, these results cannot be used as determinates of dermal absorption.

Studies in humans

Human adipose tissue studies

In a study (non-GLP) to measure residues of AHTN, two human fat samples (origin not specified) were extracted with hexane and the extracts analysed by selective ion trap GC/MS/MS technique for residues of AHTN. Residues were found in both samples at levels of 56 and 72 µg/kg fat (Eschke et al., 1995b).

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 AHTN residues by GC/MS. AHTN was found in all 14 samples at concentrations ranging from 8 to 33 µg/kg fat (mean 19 µg/kg). 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, 15 human fat samples obtained over the years 1983/4 and 1994 in Switzerland from cadavers of 10 females and 5 males (age group 3-100 years) were analysed for residues of AHTN by homogenisation followed by extraction with cyclohexane/ethyl acetate (1:1) and analysed by GC/MS. AHTN was detected in all samples with a range of 1.0 – 23 µg/kg fat (mean 9 µg/kg) (Müller et al., 1996).

Human milk studies

In a study (non-GLP) to determine residues of AHTN, two human breast milk samples (origin not specified) were extracted with hexane and the extracts analysed by selective ion trap GC/MS/MS technique for residues of AHTN. Residues were found in both samples at levels of 290 and 250 µg/kg fat or 3.1 and 1.0 µg/kg whole milk based on measured fat contents of 1.06 and 0.41%, respectively (Eschke et al., 1995b).

In a similar study (non-GLP), five breast milk samples were obtained from 4 nursing mothers in Germany and were extracted according to an AOAC method (Helrich, 1990) and analysed for AHTN residues by GC/MS. All samples contained some AHTN at concentrations ranging from 11 – 58 µg/kg milk fat (Rimkus and Wolf, 1996). The reported level of 2% milk fat is below the typical value of 3-4%.

In another larger study (non-GLP) of AHTN residues, breast 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 birth and 23.2 kg/m^2 at time of milk sampling). The protocol was designed to minimize contamination (all equipment was carefully cleaned and the breast area was cleaned 3 times with cotton swabs immersed in propylene glycol), all mothers were asked to report on their use of various household products including soaps, detergents and cosmetics as well as their consumption of fish products. A separate experiment shows that after each cleaning the cotton swabs contained considerable levels of AHTN (54-112 ng/ml). AHTN was detected in 36.4% of all samples. The maximum level reported was 0.27 µg/g milk fat

and the mean concentration 0.016 µg/g milk fat. Based on the reported mean milk fat level of 3.67%, this corresponds to a maximum level in the whole milk of 9.9 µg/kg milk with a mean of 0.59 µg/kg. There was no convincing correlation of AHTN levels in the milk with age of mother, body mass, loss of body mass and weight, time of breast-feeding, diet, or use of household products and cosmetics (Sonnichsen et al., 1999).

Dermal

In a GLP compliant study, the absorption and excretion of total radioactivity was determined in 3 human male volunteers (age 33-47 years, weight 73-81 kg). ^{14}C-AHTN (uniformly labelled in the aromatic ring – radiochemical purity >98%) in 70 % ethanol 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 eau de toilette, i.e. 0.24% in 70% ethanol. A nominal volume of 0.5 ml of the solution containing 2.4 mg ^{14}C-AHTN/ml (actual mean 1.09 mg ^{14}C-AHTN) was applied to 100 cm^2 (0.011 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% aqueous 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 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 (~ 67%) 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 4.2% 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) in two subjects and 1.1% in the third. A mean of 0.5% (range 0.2-0.8%) was excreted in the urine. Concentrations in whole blood and plasma were also below the limits of accurate measurement (*ca* 6 ng/ml and 1.04 ng/ml, respectively) at all sampling times. A further 14.5% AR was detected in dressings at the 120 hr time showing that considerable radioactivity remained in the skin after washing, which would subsequently evaporate from skin. From the recoveries in faeces and urine a mean total absorption of 0.9% (range 0.2-1.9%) could be calculated. Tape stripping at 120 hr indicated that only trace amounts (0.064%) remained in the upper layers of the stratum corneum at that time. The mean total recovery from excreta, dressings, swabs and skin strips was 86.4% AR. A separate evaporation experiment indicated that approximately 24.2% of the AHTN would be expected to evaporate under conditions of the experiment (Girkin, 1996; Ford et al., 1999).

Oral

No data available.

In vitro studies

The dermal absorption (non-GLP, but with QA-statement) of AHTN was determined over a 24-hr period according to the methodology of the SCCNFP. Radiolabeled ^{14}C-AHTN (uniformly labelled in the aromatic ring – radiochemical purity 99.6%) was applied in 1% (w/v) solution in ethanol (96% v/v) to human epidermal membranes (prepared from full-

thickness 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). There were two control cells. The area of the membrane available for absorption was approximately 1 cm^2 and the applied dose was a mean of (n=12) 20±0.4 µL/cm^2 (hence, 200 µg AHTN/cm^2). Receptor fluid (6% Volpo N20 (to enhance solubility) in pH 7.4 phosphate buffered saline) was sampled at 1, 2, 6, 12 and 24 hrs. After 24 hrs, the epidermal membranes were wiped and stripped. The amount of material absorbed into the receptor phase after 24 hr was 0.38±0.06% of the applied dose. The majority of applied AHTN (85±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 3.8±0.3% of the applied dose and the remaining stratum corneum plus epidermis 3.5±0.3% of the applied dose. As per SCCNFP guidelines, levels of AHTN in the remaining stratum corneum plus epidermis, filter paper (on which the epidermis samples rested; 0.19±0.03% of the applied dose) and permeated AHTN were combined to produce a total absorbed dose value of 4.1±0.4% of the applied dose. The evaporative loss (assessed in a separate experiment under the same conditions) over 24 hrs was 2.9% of the applied dose. Overall-recovery of radioactivity at 24 hrs was 92.5±0.7% (Green and Brain, 2001).

Summary of toxicokinetics, metabolism and distribution

The quality of the available oral absorption data are not sufficient to establish an absorption percentage of good quality but, based on urine, cage washing and tissue levels in the study by Wu (2002), absorption of at least 50% can be concluded. For oral exposure, an assumption of 50% absorption will be used in the risk characterization.

In the *in vivo* human study, under conditions simulating exposure from the fragrance use of AHTN, a 6 hr unoccluded exposure at realistic concentrations in alcoholic solution, results in absorption *into* the skin (at least 15%). 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, maximally 2% in total was actually absorbed under the conditions of this experiment. An *in vivo* study in rats supports the assumption that a good indication of the amount absorbed is the amount excreted. Although approximately 14% of the applied radioactivity was not recovered, from a separate evaporation, study it appears that under non-occlusive conditions about 24% of an applied dose may evaporate from human skin.

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 13% of the applied dose was formed after the 6-hr application with about 2 of this 13% reservoir being lost presumably from reverse diffusion and/or desquamation to the dressing after dose removal. Based on the amount remaining in the tissues (excluding that at the site of dosing, i.e. 1.5%) at sacrifice (1.7%) and the amount excreted (17.1%), almost all (14.5%) of which was in the faeces, a total absorption under the conditions of this experiment of 18.8% can be concluded. The principal differences from the human study were the much larger absorption because of the application under occlusion and the well-known fact that rat skin is more permeable than human. For risk characterisation, a value of 20% will be used for dermal absorption via rat skin. This value also accounts for a continued absorption of (part of) the skin reservoir still present at 120 hr (1.5%).

The most direct study of dermal absorption is the *in vitro* 24-hr absorption study with human epidermal membranes according to the recommendations of the SCCNFP. In this study, 0.38% of the applied dose was found in the receptor fluid after 24 hr under non-occlusive

conditions, however, 3.5% 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 4.1% of the applied dose. From a separate experiment, it appears that under the same conditions about 2.9% of an applied dose may evaporate from human skin *in vitro*. Correcting for evaporation loss would bring the skin permeation close to the results of the human *in vivo* skin absorption study. This study is the only 24-hr application and shows linear skin permeation, thus all demands on such a skin permeation study are fulfilled. As the other skin absorption studies have limitations such as 6 hr exposure time or a small number of samples, for risk characterization 4.1% as value for skin absorption will be used.

Intravenous administration of AHTN to rats and the pig results in rapid distribution. Excretion in the rat is primarily via the faeces as was seen in the dermal study (~ 76% of total excretion compared to ~84% after dermal exposure) but in the pig the principle route of excretion is via urine similar to what was seen in the human study. In neither of these studies was any evidence of accumulation seen. However, clearance from the fat was considerably slower than from other organs. It is noteworthy that in the intravenous studies, no unmetabolised AHTN is present in the urinary radioactivity. The faeces (the major excretion route of the rat) were not analysed for metabolites or parent.

An oral study with pregnant and later lactating rats shows that orally dosed AHTN can end up in the mother's milk albeit primarily as metabolites of AHTN. No evidence of accumulation was seen. The levels seen in the milk of the lactating dams can aid in the interpretation of the study where neonate rats were exposed to AHTN and its metabolites through nursing.

AHTN is found in human milk fat and in adipose tissue.

AHTN is also found in human milk where the maximum level reported was 267 μg/kg milk fat and the mean concentration was16 μg/kg milk fat (based on a measured fat content of 3.67% this translates into levels in whole milk of 9.8 and 0.59 μg/kg) and adipose tissue at levels ranging from 1 – 72 μg/kg fat.

Conclusion

AHTN has been shown to be poorly absorbed dermally and then 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 AHTN in rats and humans, values of 20 and 4.1%, respectively, are taken forward to the risk characterisation.

5.2.1.11 Experience with Human Exposure

Twenty-one patients allergic to perfumes and sweet smelling constituents were studied by patch testing with 21 substances recommended by the International Contact Dermatitis Research Group and with 57 perfume components. Fixolide (purity not reported) was tested at 3% in petrolatum. Two sensitisation reactions were observed however, the methodology was questionable and the results cannot be interpreted (Meynadier, et al. 1986).

A multicentre study with patch tests with 48 fragrance materials was reported. Tonalid (purity not reported) was tested in either a 1 or 5% solution in petrolatum on 313 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. No sensitisation was observed with Tonalid. (Frosch, et al. 1995).

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

5.2.2 Identification of critical endpoints

The oral LD_{50} values for AHTN ranged from 570-1377 mg/kg bw. The acute dermal LD_{50} values are >5000mg/kg bw.

Inhalation is not considered a significant route of exposure.

AHTN 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 no significant evidence either from animal or human studies of potential for dermal sensitisation. AHTN shows no photosensitisation potential in humans at concentrations significantly higher than would be encountered from the use of fragranced household products.

In a 28-day oral gavage study, no effects of AHTN were seen at doses up to and including 10 mg/kg bw/day.

In a 90-day study, clear mild haematological effects were seen at the highest dose administered, 50 mg/kg bw/day. These effects may be associated with observations of dark discolouration of the liver and mesenteric lymph nodes seen in most high dose animals but in no animals at lower doses. Observations in animals maintained on a treatment free regime for 28 days following the 90-day treatment period indicate that the effects are reversible. Although the differences from controls were small and generally within historical ranges seen for rats in this laboratory, the overall pattern is such it cannot be excluded that these effects are of adverse nature despite the report's conclusion that these effects "were considered to be insufficient to have compromised the animals and to be of minor toxicological significance."

Based on similar, but less obvious, haematological effects at 15 mg/kg bw/day. A NOAEL of 5 mg/kg bw/day can be concluded.

In a sub-acute study with i.p. administration, AHTN did not show peroxisomal and PB/3MC-like inducing properties.

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

In an oral peri/postnatal toxicity study (exposure of the F_1-generation to AHTN was only *in utero* during the perinatal phase or through any transfer in the milk of the lactating dams) no toxicity was seen at dose levels of 2, 6 or 20 mg/kg bw/d in the dams or their F1 and F2 offspring. 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-AHTN/kg bw per day. Levels up to 1.89 and 25 mg AHTN equivalents (i.e. AHTN + metabolites)/l of whole milk were reported, for maternal oral doses of 2 and 20 mg/kg bw/d, respectively. Actual intakes in the pups cannot be determined because milk consumption during nursing was not measured. However these levels can be compared to the average level of 0.59 ppb with a maximum of 9.8 ppb in human milk samples.

In an oral developmental study with rats, maternal toxicity occurred at 50 mg/kg bw/day. Developmental toxicity was not seen at the highest dose administered, 50 mg/kg bw/day. Therefore, the NOAEL for maternal toxicity can be established at 15 mg/kg bw/day. There is

no evidence for developmental toxicity and the developmental NOAEL is ≥50 mg/kg bw/day, the highest dose administered.

A conservative determination (*in vitro* human skin) dermal absorption study shows a 24 hr absorption of 4.1% 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 **(**8.15 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 3.4 x 10^{-5} cm/h.

The quality of the available oral absorption data are not sufficient to establish an absorption percentage of good quality but, based on urine, cage washing and tissue levels in the study by Wu (2002), 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 5 mg/kg bw/day. Based on the observation of haematological effects at 50 mg/kg bw/day and similar effects at 15 mg/kg bw/day, the NOAEL can be considered to be 5 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 AHTN.

In a rat developmental toxicity study, there was maternal toxicity as evidenced by significantly reduced weight gains at 50 mg/kg bw/day on days 7 to 10. Weight gains at 15 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 AHTN was concluded to be 15 mg/kg bw. Based on no adverse foetal effects at the highest dose administered, 50 mg/kg bw, the developmental NOAEL may be considered to be ≥50 mg/kg bw.

Because the principal route of exposure to AHTN 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 4.1% of the applied dose can be concluded. The quality of the available oral absorption data are not sufficient to establish an absorption percentage of good quality but, based on urine, cage washing and tissue levels in the study by Wu (2002), absorption of at least 50% can be concluded.

5.3 Risk Assessment

5.3.1 Margin of Exposure Calculation

The lowest NOAEL from repeated dose oral rat studies is considered to be 5 mg/kg bw/day. Based on an assumption of 50% absorption, this translates into a systemic NOAEL of 2.5 mg/kg bw/day. A highly unlikely total consumer exposure (excluding possible intake from fish) of 0.033 μg/kg bw/day (this figure is already adjusted for systemic exposure) of AHTN is estimated based on 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 75,000.

While the presence of AHTN in fish is due to all uses (including perfumes and cosmetics) the margin of safety from exposure (0.5 µg/kg bw/day) resulting from consumption of fish can be calculated to be 5,000.

Because AHTN 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 0.07 µg/kg bw/day with a maximum of 1.2 µg/kg bw/day. The maximum exposure can be compared to the NOAEL of 5 mg/kg bw/day from the rat 90-day oral study to calculate a MOS of >4000.

In an oral peri/post natal study in which female rats were exposed to AHTN 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 AHTN (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 1600 to 3600 times the mean levels and approximately 200 to 1000 times the maximum level found in human milk samples (0.59 and 9.8 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, AHTN 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 AHTN 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 AHTN 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 AHTN containing aerosols. Because of the very low volatility of AHTN and low levels of use, inhalation exposures are negligible compared to dermal. The consumer aggregate exposure to AHTN from the use of household cleaning products has been estimated to be at maximum 0.033 µg/kg bw/day.

The available toxicological data demonstrate that AHTN 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 no significant evidence either from animal or human studies of potential for dermal sensitisation. AHTN shows no photosensitisation potential in humans at concentrations significantly higher than

would be encountered from the use of fragranced consumer products. AHTN shows no significant systemic toxicity after repeated oral dosing at doses up to5 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, there was no developmental toxicity at any dose ($NOAEL_{developmental\ toxicity}$ ≥50 mg/kg bw/day), and maternal toxicity only at 50 mg/kg bw/dy, the highest dose administred ($NOAEL_{maternal\ toxicity}$ 15 mg/kg bw/day).

The comparison of the aggregate exposure and the systemic NOAEL results in a MOE of over 75,000. 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.

In summary, AHTN does not pose a risk of adverse health effects to consumers from use of its use in household cleaning products.

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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.