EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate C - Public Health and Risk Assessment
C7 - Risk assessment

SCIENTIFIC COMMITTEE ON CONSUMER PRODUCTS

SCCP

Opinion on

4-Amino-2-hydroxytoluene

COLIPA N° A27

Adopted by the SCCP
during the 9th plenary meeting of 10 October 2006

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1. BACKGROUND

Submission I, II and III for 4-amino-2-hydroxytoluene were submitted by COLIPA[1] in May 1983, January 1988 and February 1991 respectively.

The Scientific Committee on Cosmetology (SCC) adopted at its 54th plenary meeting of 10 December 1993 an opinion with the "*comment: Several investigations that might have been expected have not, apparently, been carried out. Perhaps in view of the ambiguous results of the tests for mutagenicity a test for carcinogenicity might be required. Tests in man for sensitization, photosensitivity and percutaneous absorption might be desirable. Other tests, such as for teratogenicity, or reproduction tests, might or might not be thought desirable. SCC requires a study on chromosome aberration test in mammalian cells in vitro*".

The substance is currently regulated as an oxidative hair dye by the Cosmetics Directive (76/768/EC), Annex III, part 2 under entry 35 on the List of provisionally allowed substances, which cosmetic products must not contain except subject to restrictions and conditions laid down.

According to the current submission IV, submitted by COLIPA in July 2005, 4-amino-2-hydroxytoluene it is used as a precursor in oxidative hair dying products with of maximum concentration on the scalp of 1.5%.

Submission IV presents updated scientific data on the above mentioned substance in line with the second step of the strategy for the evaluation of hair dyes (http://europa.eu.int/comm/enterprise/cosmetics/doc/hairdyestrategyinternet.pdf) within the framework of the Cosmetics Directive 76/768/EEC.

2. TERMS OF REFERENCE

Does the Scientific Committee on Consumer Products (SCCP) consider 4-amino-2-hydroxytoluene safe for consumers, when used in oxidative hair dye formulations with a concentration on the scalp of maximum 1.5% taking into account the scientific data provided?

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association

3. OPINION

3.1. Chemical and Physical Specifications

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

4-Amino-2-hydroxytoluene (INCI)

3.1.1.2. Chemical names

5-amino-2-methylphenol (IUPAC)
Phenol, 5-amino-2-methyl- (CA INDEX NAME, 9CI)
5-Amino-o-cresol

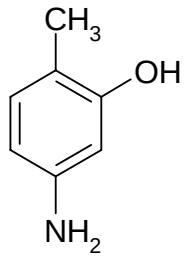
3.1.1.3. Trade names and abbreviations

Jarcol 2M5AP
Rodol PAOC
HAARPURPUR 23032 WR

3.1.1.4. CAS / EINECS number

CAS: 2835-95-2
EINECS: 220-618-2

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: C_7H_9NO

3.1.2. Physical form

Beige to brown crystalline powder

3.1.3. Molecular weight

Molecular weight: 123.16 g/mol

3.1.4. Purity, composition and substance codes

Purity and impurities in various batches

Description	**Batch**					
	20010504 (R0068430)	**A27 (14.5.90) King & Harnasch**	**41128**	**20000502 (R00056990)**	**20000506 (R00056995)**	**AR 800 (B177)**
Identification/ Characterisation	NMR, HPLC					
NMR content % w/w	97.6	97.9	98.2	99.9	99.7	99.5
HPLC purity, area % **210 nm** **254 nm** **274 nm**	 99.9 99.9 99.9		 99.9 99.9 99.9	 99.9 99.9 99.9	 99.9 99.9 (285 nm)	 99.7 99.6 99.9
Loss on drying[a], % w/w	0.95	0.73	0.2	1.4	-	0.2
Water content, % w/w	0.01	0.15	-	<0.05	0.02	0.04
Sulphated ash, % w/w	0.01	0.01	<0.1	0.01	0.01	0.11
Potential impurities						
2,4-diaminotoluene, ppm	1	2	3	26	Ca. 5	35
2-methyl-5-nitroaniline, ppm	<10*	<10	<10*	<10*	<10*	<10*
2-methyl-5-nitrophenol, ppm	<1	n.d.	<1	<1	<1	n.d.
o-Toluidine, ppm	<10*	13	<10*	<10*	<10*	<10*
Organic solvents	Methanol, ethanol, isopropanol, n-propanol, acetone, ethylacetate, cyclohexane, methyl ethyl ketone and monochlorobenzene were not detected at 100 ppm detection limit					

[a] substance sublimes
* detection limit
n.d. not done because of lack of substance

3.1.5. Impurities / accompanying contaminants

See 3.1.4

3.1.6. Solubility

Water: 4.112 g/l (Method: EU A6)
DMSO: > 100 g/l
Ethanol: 40 - 80 g/l

3.1.7.	Partition coefficient (Log P_{ow})

Log P_{ow} : - 0.53 at pH ca. 7.22, room temperature (Method: EU –A8)

3.1.8.	Additional physical and chemical specifications

Organoleptic properties:

Melting point:	160-161°C
Boiling point:	Decomposition at 236°C
Flash point:	Relative self-ignition temperature (EU A.16) >105°C
Vapour pressure:	8.5 exp -5 hPa (20°C)
Density:	1.244)
Viscosity:	/
pKa:	/
Refractive index:	/

3.1.9.	Stability

5% solution of 4-Amino-2-hydroxytoluene in acetone/water (1:1) was stable (deviation ±0.5% from original concentration), when stored for 2 hours at room temperature in the absence of light.

5% solution of 4-Amino-2-hydroxytoluene in DMSO (1:1) was stable (deviation ±0.3% from original concentration), when stored for 2 hours at room temperature in the absence of light.

General comments to physico-chemical characterisation

* Stability of 4-amino-2-hydroxytoluene in marketed products is not reported

3.2. Function and uses

4-Amino-2-hydroxytoluene is used in oxidative hair dye formulations at a final concentration of 1.5%, after mixing with peroxide developer.

3.3. Toxicological Evaluation

3.3.1.	Acute toxicity

3.3.1.1.	Acute oral toxicity

Guideline: /
Species/strain: rat, CFY

Test substance:	4-amino-2-hydroxytoluene
Batch:	not specified
Purity:	not specified
Vehicle:	10% suspension in aqueous gum tragacanth (0.5%) containing sodium sulphite (0.05%)
GLP:	not in compliance

Rats, 4 per sex/dose were administered the test substance via gavage in dose levels of 0, 0.4, 1, 2.5 or 6.4 g/kg bw. To determine the median lethal dose more precisely dosing was extended to a larger group, and additional groups of 5 rats per dose/sex, were administered the test substance via gavage in dose levels of 0, 1.6, 2.5, 4.0 or 6.4 g/kg bw. Shortly after dosing clinical signs observed were lethargy, piloerection and decreased respiration rate. Above 1.6 g/kg these signs were accompanied by ataxia, and in rats dosed above 2.5 mg/kg also fine body tremors and increased lacrimation was observed.
The acute median lethal oral dose of 4-amino-2-hydroxytoluene in rats was calculated to be 3.6 (95% confidence limits: 3.1-4.0) g/kg bw.

Ref.: 3

Comment
Despite the deficiencies of this study (purity and batch number unknown, not according to a guideline) this study is useful for evaluation.

3.3.1.2. Acute dermal toxicity

No data submitted

3.3.1.3. Acute inhalation toxicity

No data submitted

3.3.2. Irritation and corrosivity

3.3.2.1. Skin irritation

Guideline:	/
Species/strain:	Rabbit, strain Albino
Group size:	3, sex not indicated
Test substance:	4-amino-2-hydroxy toluene
Batch:	/
Purity:	/
Dose:	0.5 ml, 2.5% solution of 4-amino-2-hydroxy toluene in 0.5% aqueous gum tragacanth containing 0.05% sodium sulphite (pH: 7.0)
GLP:	not in compliance

0.5 ml of a 2.5% solution of 4-amino-2-hydroxy toluene in aqueous gum tragacanth (0.5%) containing 0.05% sodium sulphite was applied to shaved areas (about 6.45 cm^2) of the back of 3 albino rabbits. After 24 h under occlusive conditions, the test item was removed. Animals were examined for signs of erythema and oedema 24 and 72 hours post exposure period.

Results
None of the animals showed any observable response to treatment throughout the 72 hours observation period.

Conclusion
Under 24 hour occlusive contact, a 2.5% aqueous solution of 4-amino-2-hydroxy toluene showed no skin irritating potential in rabbits under the described test conditions. Although the test procedure did not follow currently accepted guidelines and the purity of the test substance is unknown, the results can be accepted as being indicative of the irritant potential of a 2.5% dilution of the test substance.

Ref.: 14

3.3.2.2. Mucous membrane irritation

Guideline:	/
Species/strain:	Rabbit, strain Albino
Group size:	3, sex not indicated
Test substance:	4-amino-2-hydroxy toluene
Batch:	/
Purity:	/
Dose:	0.1 ml 2.5% solution of 4-amino-2-hydroxy toluene in 0.5% aqueous gum tragacanth containing 0.05 % sodium sulphite (pH: 7.0)
GLP:	not in compliance

0.1 ml of 2.5% solution of 4-amino-2-hydroxy toluene in 0.5% aqueous gum tragacanth containing 0.05% sodium sulphite, was introduced into the conjunctival sac of one eye of 3 albino rabbits, the untreated eye served as control. 10 seconds after instillation of the test material, the eyes were washed with 50 ml (37°C) water. Both eyes were examined at 1, 24, 48 and 72 h post application and effects were scored according to the Draize scheme.

Results
No effects on the cornea or iris were noted at any reading. In all animals, a slight conjunctival redness (score 1) was noted 1 hour after instillation but this redness had gone within 24 hours after application in all animals.

Conclusion
Based on the slight and reversible effects noted 1 hour post instillation in this rabbit study, a 2.5% aqueous solution of 4-amino-2-hydroxy toluene showed transient irritation to rabbit conjunctivae under the test conditions. Although the test procedure did not follow currently accepted guidelines and the purity of the test substance is unknown, the results can be accepted as being indicative of the irritant potential of a 2.5% dilution of the test substance.

Ref.: 15

3.3.3. Skin sensitisation

Local Lymph Node Assay (LLNA), test 1

Guideline:	OECD 429 (2002)
Species:	Mouse, strain CBA/Ca01aHsd

Group size:	5 females per test concentration
Test substance:	HAARPURPUR 23032 WR
Batch:	41128
Purity:	98.2%
Concentrations:	0.5, 1.5, 3 and 5 % in aqua/acetone (1:1) mixed with olive oil at a ratio of 4:1
GLP:	in compliance

25 µl of 0 (vehicles only), 0.5, 1.5, 3 and 5 % HAARPURPUR 23032 WR in a mixture of aqua/acetone (1:1) with olive oil (4:1) were applied to the surface of the ear of five female mice per group for three consecutive days. As a positive control, p-phenylenediamine (PPD) at 1% in acetone/aqua/olive oil was investigated in parallel under identical test conditions.

At day 5, the mice received an intravenous injection of 250 µl solution containing 20 µCi of [H3] methyl thymidine. Approximately five hours later, the mice were killed, and the draining auricular lymph nodes were removed and collected in PBS. After preparing a single cell suspension for each mouse, cells were precipitated by TCA, and the radioactivity was determined (incorporation of [H3] methyl thymidine in the pellets) by means of liquid scintillation counting as disintegration per minute (dpm).

Results
The stimulation indices in the groups with acetone/aqua/olive oil used as vehicle were:

3.2 (0.5% test group)
5.9 (1.5% test group)
5.3 (3.0% test group)
9.4 (5.0% test group)

With acetone/aqua/olive oil as vehicle, the skin sensitising potential of HAARPURPUR 23032 WR was observed. An EC3 was estimated by extrapolation to be 0.44%.
The sensitivity of the test system was demonstrated by the reaction of the positive control p-phenylenediamine (1%) which exhibited a stimulation index of 31.2.

Conclusion
HAARPURPUR 23032 WR produced a biologically relevant immune response in local lymph nodes after dermal application even at the lowest test concentration of 0.5%. As an EC3 of 0.44% is calculated by linear extrapolation, HAARPURPUR 23032 WR is a strong skin sensitiser.

Ref.: 16

Local Lymph Node Assay (LLNA), test 2

Guideline:	OECD 429 (2002)
Species:	Mouse, strain CBA/Ca01aHsd
Group size:	5 females per test concentration
Test substance:	HAARPURPUR 23032 WR
Batch:	41128
Purity:	98.2%
Concentrations:	0.5, 1.5, 3 and 5% in DMSO

GLP: in compliance

25 µl of 0 (vehicles only), 0.5, 1.5, 3 and 5% HAARPURPUR 23032 WR in DMSO were applied to the surface of the ear of five female mice per group for three consecutive days. As a positive control, p-phenylenediamine (PPD) at 1% in DMSO was investigated in parallel under identical test conditions.

At day 5, the mice received an intravenous injection of 250 µl solution containing 20 µCi of [H3] methyl thymidine. Approximately five hours later, the mice were killed and the draining auricular lymph nodes were removed and collected in PBS. After preparing a single cell suspension for each mouse, cells were precipitated by TCA, and the radioactivity was determined (incorporation of [H3] methyl thymidine in the pellets) by means of liquid scintillation counting as disintegration per minute (dpm).
The mean dpm per treated group was determined, and the stimulation index (test item compared to the concurrent vehicle control) was calculated.

Results
The stimulation indices in the groups with DMSO used as vehicle were:

2.6 (0.5% test group)
2.4 (1.5% test group)
2.8 (3.0% test group)
3.9 (5.0% test group)

With DMSO as vehicle, a skin sensitising potential of HAARPURPUR 23032 WR was noted. An EC3 value (equal to the concentration inducing a stimulation index of 3) of 3.4 was calculated for HAARPURPUR 23032 WR under the given test conditions.
The sensitivity of the test system was demonstrated by the reaction of the positive control p-phenylenediamine (1%) which exhibited a stimulation index of 12.7.

Conclusion
HAARPURPUR 23032 WR induced a biologically relevant immune response in local lymph nodes after dermal application to the mouse ear in the test concentration range of up to 5.0%. HAARPURPUR 23032 WR is concluded to be a moderate skin sensitiser under the described test conditions in DMSO, with a calculated EC3 value of 3.4.

Ref.: 17

Other information on skin sensitisation

4-Amino-2-hydroxytoluene was investigated for skin sensitising properties in guinea pigs (1976 and 1979) as the information submitted to the SCC in 1993.

In the first test, a 3% aqueous solution (containing 2% Natrosol 250HR, 2% Tween 80, 10% isopropanol and 0.05% sodium sulphite) was assessed in an open epicutaneous test, in which the test item was applied daily for a total of 3 weeks (induction) to 19 guinea pigs. Challenge took place two weeks after the last induction treatment utilising the same test concentration. 4 animals showed evidence of a skin sensitising potential.

In the second test according the Magnusson Kligman protocol, 1% and 25% solutions in propylene glycol were used for the intradermal and epidermal applications. For the epidermal challenge, 25% in propylene glycol was also applied. 4 of the 10 female albino Hartley guinea pigs showed evidence of a skin sensitising potential.

Neither of these studies was performed in compliance with GLP and in at least one test a non-standardised method was used and unspecified material was tested.

Ref: none provided

3.3.4.	Dermal / percutaneous absorption

Percutaneous absorption *in vitro*

Guideline:	OECD 428 (2004)
Tissue:	Porcine skin (thickness: mean approximately 1000 µm)
Test substance:	4-amino-2-hydroxytoluene
Batch:	CFQ14292 (14C-labelled substance); 20000506 (non-labelled substance)
Purity:	> 92.9% by radio-HPLC (^{14}C-labelled substance) 99.69% non-labelled substance
Dose levels:	a) 1.5 mg/cm^2 (0.1636 mCi/ml specific activity) in an oxidative hair dye formulation with hydrogen peroxide and a reaction partner. b) 1.5 mg/cm² and the same study design as described above, but in a non-oxidative formulation without hydrogen peroxide or reaction partner.
No. of chambers:	6 (from 2 female and 1 male donors) for experiment a) oxidative 4 (from 1 donor) for experiment b) non-oxidative
GLP:	in compliance

The skin absorption of 4-amino-2-hydroxytoluene was investigated with pig skin (approximately 1000 µm thick, from 3 donors) in an oxidative formulation with hydrogen peroxide and reaction partner (equimolar amounts of toluene-2,5-diamine sulphate). 400 mg of this final formulation containing 1.5% 4-amino-2-hydroxytoluene (equivalent to the maximum use concentration) was applied once to the skin samples (4 cm²) in a typical cream formulation for oxidative hair dyes.

Diffusion chambers were used. The receptor solution (physiological phosphate buffer saline containing 100 IU penicillin/ml, 76 IU streptomycin/ml and 3 % ethanol) was pumped through the receptor chamber at a rate of 5 ml/h. The integrity of the skin was monitored at the beginning of the experiment using tritiated water.

Thirty minutes after substance application, the test item was removed by washing the skin twice with 4 ml water, then once with 4 ml of a shampoo-formulation (diluted to approximately 14%), and again twice with water. The rinsing solutions were combined and the amount of dye was determined by radioanalysis. Fractions of the receptor fluid were collected after 16, 24, 40, 48, 64 and 72 hours. At termination of the experiment, the skin was heat-treated and the "upper skin" (stratum corneum and upper stratum germinativum) was mechanically separated from the "lower skin" (lower stratum germinativum and upper dermis). Both skin compartments were extracted separately and the dye content was quantified by means of a scintillation counter.

Results

In the experiment under oxidative conditions, the majority of the test substance was recovered in the rinsing solutions (1.298 ± 0.032 mg/cm^2). Small amounts of 4-amino-2-hydroxytoluene were detected in the upper skin (2.317 ± 0.626 µg/cm^2), in the receptor fluid (2.015 ± 0.438 µg/cm^2) and in the lower skin (0.746 ± 0.547 µg/cm^2) after the 72 hour experimental period. By combining the amounts found in the receptor fluid and in the lower skin, a skin penetration rate of 2.761 ± 0.985 µg/cm^2 was obtained. The mass balance (total recovery) was between 97.3 and 99.4% for all 6 skin samples.

The maximum absorption observed in the experiment was 3.48 µg/cm^2 and this will be used for calculating the MoS.

In the experiment under non-oxidative conditions, a maximum amount of 2.59 ± 1.21 µg/cm² of 4-amino-2-hydroxytoluene and considered to be biologically available. The experimental data was not provided with in the dossier.

Conclusion
Under the described test conditions, a maximum absorption of 3.48 µg/cm^2 was observed when 1.5mg/cm^2 4-amino-2-hydroxytoluene was applied under oxidative conditions in a typical hair dye formulation.

Ref.: 19

3.3.5. Repeated dose toxicity

3.3.5.1. Repeated Dose (28 days) oral / dermal / inhalation toxicity

No data submitted

3.3.5.2. Sub-chronic (90 days) oral / dermal / inhalation toxicity

A 13 week study in rats was carried out using dose levels of 300, 900 and 2700 mg/kg bw/day.
The main toxic effects in this study were local irritative effects on the stomach mucosa, hepatotoxicity and toxic effects on the red blood cells, apart from transient effects on the general behaviour immediately after application in the beginning of the study. Although of less severity, some of these effects were also observed at the lowest dose level.
Therefore, an additional 90-day oral toxicity study was initiated to investigate lower dose levels.

Ref.: 21

13-week study in rats

Guideline:	/
Species/strain:	Rat, strain Him:OFA (SPF)
Group size:	20 animals per sex and dose
Test substance:	4-amino-2-hydroxytoluene
Batch:	851 (lot: 13/06/1977)
Purity:	91.5% (HPLC)
Dose levels:	20, 60 and 180 mg/kg bw/day
Vehicle:	0.1% gelatin 25000 swelling
Route:	Oral, gavage

Dosing schedule 13 weeks, 5 days/week
GLP: /

Doses of 20, 60 and 180 mg/kg bw/day of 4-amino-2-hydroxytoluene were administered 5 days/week to Him:OFA (SPF) rats orally by gavage over a period of 13 consecutive weeks. A control group was treated similarly with the vehicle only. The dose volume in treated and control groups was 10 ml/kg body weight.
The groups comprised 20 animals per sex which were sacrificed after 13 weeks of treatment. The dose levels of this main study were chosen on the basis of the results noted in a previous 13-week toxicity study in rats (Ref. 21). Test solutions were freshly prepared in water each day and 0.1%-Gelatin was added prior to administration for suspension purposes (indicated by the product name Relatin-25000-swelling).
Mortality and clinical signs were recorded at least once daily; body weights, water and food consumption were recorded weekly during the treatment period.
Clinical laboratory investigations (haematology, blood/clinical biochemistry) as well as urinalysis were carried out before the start of administration and twice during the treatment period. All animals were subjected to a detailed necropsy and a number of organs (kidneys, adrenals, spleen, testes, heart, liver, brain and pituitary gland) were weighed; numerous tissues and organs were fixed and stored for further examination if required.

Results
The stability, homogeneity and correctness of the dosing solutions were not analytically verified.
There were no substance-related deaths during the treatment period.
No substance-related clinical signs were noted and no significant deviations from the controls in body weight gain were found. All treatment groups revealed differences in food consumption compared to the control, but without consistent trend.
There was no substantial difference in water-consumption. No significant differences in the reticulocyte counts or other haematological examinations between the dose groups were found. Generally, no differences between the dose groups concerning urinalysis were noted. Incidental variations were noted in different dose groups concerning the density and pH, but this is not considered to represent a substance-related effect.
Gross necropsy revealed bloody soaking of the mandibular lymph nodes as the most frequently observed alteration. This can be explained by the blood sampling from the retroorbital venous plexus, a tributary region associated with this lymph node. Other than some isolated cases, no further pathological changes were noted.
For both the absolute and relative organ weights, no significant differences compared to controls were observed for any organ. At necropsy, no treatment related effects were noted.

Conclusion
4-Amino-2-hydroxytoluene orally administered for 90 days to rats at doses of 20, 60 and 180 mg/kg bw/day revealed no treatment related effects. None of the adverse effects observed in a previous experiment (Reference: 21) were detected at any of the doses administered. Under the experimental conditions of this 90-day oral toxicity study, the NOAEL for 4-amino-2-hydroxytoluene is determined to be 180 mg/kg bw.

Ref. 20

Comment
No rationale was given for the dose regimen (5 days/week).

This study is relatively old (1979) and does not comply with GLP. However, the results are useful for evaluation.

3.3.5.3. Chronic (> 12 months) toxicity

No data submitted

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1. Mutagenicity / Genotoxicity *in vitro*

Bacterial gene mutation assay

Guideline:	OECD 471
Species/strain:	*Salmonella typhimurium*, TA98, TA100, TA1535, TA1537, TA102
Replicates:	Two independent tests with and without S9 mix (plate incorporation test and pre-incubation test)
Test substance:	4-Amino-2-hydroxytoluene (WR23032) in DMSO
Batch:	20000506
Purity:	NMR 99.7% (weight)
Concentrations:	33-5000 µg/plate with and without S9 mix (6 concentrations)
GLP:	in compliance

The test substance was assessed for the induction of revertant mutations in various strains of *Salmonella typhimurium* with and without S9 mix containing phenobarbital/beta-naphthoflavone -induced rat liver postmitochondrial fraction. The test was performed according to the OECD guidelines and GLP. Concentrations were chosen on a basis of preliminary tests.

Results
Normal background growth was observed at all conditions up to 5000 µg/plate, indicating no toxicity of the test substance to the bacteria. No biologically relevant increase in revertant colony numbers was observed in any tester strain following treatment with A 27. No evidence of a dose-response was either seen. Positive controls showed a distinct increase in revertant colonies.

Conclusions
Under the experimental conditions reported, the test substance was not mutagenic to *Salmonella typhimurium*.

Ref.: 22

***In vitro* mammalian cell gene mutation test**

Guideline:	OECD 476
Cells:	L5178Y mouse lymphoma cells *($tk^{+/-}$ locus)*
Replicates:	two independent tests with and without S9 mix
Test substance:	5-Amino-2-methylphenol (23032)
Batch:	20000502
Purity:	Not given

Concentrations:	5, 10, 25, 50, 100, 250, 500 and 1000 µg/ml with metabolic activation; 1, 2.5, 5, 10, 25, 50, 100, and 250 (500 and 1000 µg/ml were toxic) without metabolic activation
GLP:	in compliance

5-Amino-2-methylphenol was investigated for the induction of gene mutations at the thymidine kinase locus in L5178Y mouse lymphoma cells with and without metabolic activation. Liver S9 fraction from Aroclor1254-induced rats was used as the exogenous metabolic activation system. Test concentrations were based on the level of toxicity. Negative and positive controls were in accordance with the OECD guideline.

Results
For the highest concentration 1000 µg/ml examined for toxicity, relative total growth was 35.58% with metabolic activation. Without metabolic activation, the highest concentration analyzed was 250 µg/ml (relative total growth 17.32%), as 500 and 1000 µg/ml were highly toxic. No significant increases in mutation frequencies by the test substance were seen with or without metabolic activation. Positive control treatments induced a clear increase in mutants.

Conclusions
Under the experimental conditions studied 5-amino-2-methylphenol was not mutagenic in mouse lymphoma L5178Y cells *(tk$^{+/-}$ locus) in vitro* with or without metabolic activation.

Ref.: 23

In vitro mammalian cell gene mutation test

Guideline:	OECD 476
Cells:	L5178Y mouse lymphoma cells *(tk$^{+/-}$ locus)*
Replicates:	two independent tests with and without S9 mix
Test substance:	5-Amino-2-methylphenol (23032)
Batch:	20000502
Purity:	Not given
Concentrations:	50, 100, 250, 500, 600, 700, 800, 900, 1000, 1250, and 1500 µg/ml with metabolic activation; 2.5, 5, 10, 25, 50, 100, 150, 200, 300, 400 and 500 µg/ml without metabolic activation
GLP:	In compliance

5-Amino-2-methylphenol was investigated for the induction of gene mutations at the thymidine kinase locus in L5178Y mouse lymphoma cells with and without metabolic activation. Liver S9 fraction from Aroclor1254-induced rats was used as the exogenous metabolic activation system. Test concentrations were based on the level of toxicity. Negative and positive controls were in accordance with the OECD guideline.

Results
For the highest concentration 1500 μg/ml examined for toxicity, relative total growth was 22.56% with metabolic activation. Without metabolic activation, the highest concentration analyzed (500 μg/ml) resulted in a relative total growth of 19.17%. The test agent induced a dose-dependent increase in mutations without metabolic activation. With metabolic activation, an increase in mutant frequency was also seen, but it was hold on a plateau and decreased at the highest doses. Colony size suggested clastogenic effects. Positive control agents induced a clear increase in mutant frequency.

Conclusions
Under the experimental conditions studied 5-amino-2-methylphenol was mutagenic in mouse lymphoma L5178Y cells *in vitro* especially without metabolic activation.

Ref.: 24

***In vitro* micronucleus test**

Guideline:	OECD draft guideline 487
Cells:	Human lymphocytes (pooled blood of two female donors)
Replicates:	Two independent tests with and without S9 mix, 24 and 48 hours after PHA stimulation
Test substance:	4-Amino-2-hydroxytoluene (WR23032) in DMSO
Batch:	20000506
Purity:	99.9% (HPLC)
Concentr. Scored:	Experiment I: 206.7, 323.0, 788.5, and 985.6 μg/ml without metabolic activation for 20 h, 24 after PHA stimulation, 28-h recovery; 788.5, 985.6, and 1232 μg/ml with metabolic activation, for 3 h, 24 after mitogen stimulation, 45-h recovery. Experiment II: 504.7, 788.5, and 1232 μg/ml without metabolic activation for 20 h, 48 h after PHA stimulation, 28-h recovery; 788.6, 985.7, 1232 μg/ml with metabolic activation for 3 h, 48 h after mitogen stimulation, 45-h recovery
GLP:	in compliance
Comment:	The OECD draft guideline 487 does not suggest a protocol with a 45-h recovery when the treatment was performed 48 h after mitogen stimulation.

4-Amino-2-hydroxytoluene was investigated for the induction of micronuclei in cultured human lymphocytes 24 h (experiment I) and 48 h (experiment II) after mitogen stimulation. The experiments were performed with and without liver S9 fraction from Aroclor1254-induced rats as an exogenous metabolic activation system. Concentrations were selected on the basis of preliminary tests. Toxicity was determined by measuring reduction in replication index (RI). Negative and positive controls were used in accordance with the draft of the OECD guideline.

Results
In the first experiment, the highest concentrations analyzed induced 67% reduction in replication index without S9 mix (985.6 μg/ml) but only a 12% reduction in replication index with S9 mix (1232 μg/ml). Without metabolic activation, a statistically significant increase in the frequency of micronucleated cells was seen at the lowest three concentrations, but not at the highest (toxic) concentration. No increase in micronuclei was seen with S9 mix. In the second experiment, the

highest concentration analyzed (1232 µg/ml) induced 61% and 52% reduction in replication index without and with S9 mix, respectively. 4-Amino-2-hydroxytoluene induced a statistically significant increase in micronuclei both with and without S9 mix, with a clear dose-response without S9 mix.

Conclusions
4-Amino-2-hydroxytoluene induced micronuclei in human lymphocytes with and without metabolic activation; a clearer response was seen without metabolic activation especially when treatment was started 48 h rather than 24 h after mitogen stimulation.

Ref.: 25

Comet assay for DNA strand breakage *in vitro*

Guideline:	According to recommendations of Tice et al. (2002)
Cells:	Chinese hamster V79 cells
Replicates:	Two separate experiments, both with and without metabolic activation.
Test substances:	4-Amino-2-hydroxytoluene (WR23032) in PEG 400 and its metabolite 4-acetylamino-2-hydroxytoluene (WR803389)
Batch:	20000506 (WR23032); MOR0877/1A (WR803389)
Purity:	HPLC 99.9% area (WR23032); HPLC 97% area (WR803389)
Concentrations:	WR23032: 308, 616 and 1232 µg/ml, WR803389: 413, 826 and 1652 µg/ml; for 3 h with and without metabolic activation
GLP:	not in compliance

4-Amino-2-hydroxytoluene was investigated for the induction of DNA strand breaks in Chinese hamster V79 cell *in vitro* with and without metabolic activation by S9 mix prepared from the livers of Aroclor 1254 -induced rats. Maximum concentration used se was limited by the molecular weight of the test substances (10 mM recommended highest concentration in most *in vitro* tests). Cell toxicity was assessed by counting viable cells.

Results
In the absence of metabolic activation, both test items caused a slight cytotoxic effect, reducing cell viability to 90.5 (WR23032) and 93.7 % (WR803389). The relative cell density was reduced to 84.4 and 87.6 % (respectively). In the presence of metabolic activation, both test items caused a slight cytotoxic effect, reducing the viability to 92.0 and 92.3 % (respectively). The relative cell density was reduced to 91.6 and 89.8 % (respectively). 4-Amino-2-hydroxytoluene induced a clear dose-dependent increase in DNA damage of the cells, none of the tested dose levels of its main metabolite 4-acetylamino-2-hyroxytoluene showed a biologically relevant increase in DNA damage as compared with corresponding vehicle controls. Appropriate reference mutagens were used as positive controls and led to a distinct increase of DNA damage, indicating that the experiments were sensitive and valid.

Conclusion
Under the test conditions reported, 4-amino-2-hydroxytoluene but not 4-acetylamino-2-hydroxytoluene induced DNA damage in Chinese hamster V79 cells *in vitro*, as measured by the comet assay.

Ref.: 42

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Mouse bone marrow micronucleus test

Guideline:	OECD 474
Species/strain:	Mouse, NMRI
Group size:	5 males + 5 females
Test substance:	4-Amino-2-hydroxytoluene (23032) in water
Batch:	20000502
Purity:	Not given
Dose levels:	20, 100, and 200 mg/kg bw (once intraperitoneally)
Sacrifice time:	24 and 48 (highest dose group only) h after the treatment
GLP:	in compliance

4-Amino-2-hydroxytoluene was investigated for the induction of micronuclei in the bone marrow polychromatic erythrocytes of mice. Negative and positive controls were in accordance with the OECD guideline. Dose selection was based on a dose range-finding assay where 200 mg/kg bw i.p. was observed to induce signs of toxicity. The test article was formulated in water and administered once. The proportion of polychromatic erythrocytes among all erythrocytes was used a measure of bone marrow toxicity.

Results
No increase in micronucleated polychromatic erythrocytes by 4-amino-2-hydroxytoluene was noted in the experiments. No clear dose-related decreases in the proportion of polychromatic erythrocytes were seen. Thus it was left unclear if the substance reached bone marrow. However, systemic availability can be assumed because of the application route used. The positive control treatment (cyclophosphamide) induced a clear increase in micronucleated cells.

Conclusion
Under the test conditions reported, 4-amino-2-hydroxytoluene did not induce micronuclei in bone marrow polychromatic erythrocytes of mouse. It did not, however, induce bone marrow toxicity.

Ref.: 27

Mouse bone marrow micronucleus test

Guideline:	OECD 474
Species/strain:	Mouse, NMRI
Group size:	5 males + 5 females
Test substance:	4-Amino-2-hydroxytoluene (WR23032) in PEG 400
Batch:	20000506
Purity:	HPLC 99.7% weight
Dose levels:	125, 250, and 500 mg/kg bw (once orally)
Sacrifice time:	24 and 48 (highest dose group only) h after the treatment
GLP:	in compliance

4-Amino-2-hydroxytoluene was investigated for the induction of micronuclei in the bone marrow polychromatic erythrocytes of mice after oral dosing. Negative and positive controls

were in accordance with the OECD guideline. Dose selection was based on pre-experiments where the two highest doses used in the actual study showed signs of systemic toxicity. The test article was formulated in PEG 400 and administered once. The proportion of polychromatic erythrocytes among all erythrocytes was used a measure of bone marrow toxicity.

Results
No increase in micronucleated polychromatic erythrocytes by 4-amino-2-hydroxytoluene was noted in the experiments. No clear dose-related decreases in the proportion of polychromatic erythrocytes were seen, but urine was discoloured orange, which was considered an indication of systemic distribution and bioavailability of the test substance. The positive control treatment (cyclophosphamide) induced a clear increase in micronucleated cells.

Conclusion
Under the test conditions reported, 4-amino-2-hydroxytoluene did not induce micronuclei in bone marrow polychromatic erythrocytes of mouse.

Ref.: 28

Comment
The toxicokinetic data indicate a good oral absorption.

Comet assay for DNA strand breakage in rats *in vivo*

Guideline:	According to recommendations of Hartmann et al.
Species/strain:	Rat, Wistar; liver, stomach, urinary bladder epithelium
Group size:	1st experiment: 5 males, 2nd experiment: 10 males
Test substance:	4-Amino-2-hydroxytoluene (WR23032) in PEG 400
Batch:	20000506
Purity:	99.7%
Dose levels:	1st experiment: 500, 1000, 2000 mg/kg bw (twice, 20 h apart, by gavage); 2nd experiment: 2000 mg/kg (twice, 20 h apart, by gavage)
Sacrifice time:	3 h after the last dosing
GLP:	in compliance

4-Amino-2-hydroxytoluene was investigated for the induction of DNA strand breaks in the liver, stomach and urinary bladder epithelium of Wistar rats after two treatments by gavage (in polyethylene glycol, PEG 400), 20 h apart. DNA migrated out from the nucleus in single cell gel electrophoresis was used as a measure of DNA strand breakage. Negative and positive controls were included. Histopathology of the liver, stomach, and urinary bladder was assessed in a parallel experiment. As the first experiment gave unclear results, the experiment was repeated with the highest dose, using twice as many animals.

Results
Hepatotoxicity was demonstrated histopathologically at 2000 mg/kg bw of 4-amino-2-hydroxytoluene. Statistically significant increases in mean tail length, mean tail moment, and mean tail intensity were seen at 500 mg/kg bw and for mean tail moment at 1000 mg/kg bw of 4-amino-2-hydroxytoluene. Hepatotoxicity and loss of cells may in theory explain why no DNA damage was seen at 2000 mg/kg bw. Considering both experiments together, no biologically relevant increase in various comet assay parameters were seen in the stomach and urinary bladder. The positive control treatment induced a clear increase in comet parameters.

Discoloured urine, piloerection, or narrowed palpebral fissure were considered to demonstrate relevant systemic exposure.

Conclusion
Under the reported test conditions (oral exposure), primary genotoxicity of 4-amino-2-hydroxytoluene to rat liver could not be excluded. No genotoxicity was seen in rat stomach or urinary bladder.

Ref.: 30

In vivo unscheduled DNA synthesis (UDS) test in rats

Guideline:	OECD 486
Species/strain:	8 to 11 week old male Sprague-Dawley rats
Group size:	Three rats evaluated, three slides scored per rat per dose level, vehicle and positive controls at 2 to 4 hours and 12 to 16 hours of exposure.
Test substance:	4-Amino-2-hydroxytoluene (WR23032) in PEG 400
Batch:	20000506
Purity:	99.9%
Dose levels:	500, 1000, and 2000 mg/kg bw, single oral gavage at 10 ml/kg bw dosing volume; for 2-4 h or 12-16 h.
GLP:	In compliance

4-Amino-2-hydroxytoluene was investigated for the *in vivo* induction of DNA damage, as measured by unscheduled DNA synthesis, in hepatocytes of rats. Negative and positive controls were included according to the guidelines. A toxicity/histopathology test was performed in parallel.

Results
The test article, WR23032, did not induce a significant increase in the mean number of net nuclear grain counts (i.e., an increase of at least 5 counts over the negative control group) in hepatocytes isolated either 2 to 4 hours or 12 to 16 hours after dose administration.

Conclusion
Under the test conditions reported, 4-amino-2-hydroxytoluene was negative in the *in vivo* unscheduled DNA synthesis (UDS) test in rats.

Ref.: 31

Comment
The toxicokinetic data indicate a good oral absorption.

3.3.7. Carcinogenicity

No data submitted

3.3.8. Reproductive toxicity

3.3.8.1. One generation reproduction toxicity

Guideline:	OECD guideline no. 415 (1983)
Species/strain:	Rat, strain Wistar Crl:(WI) BR
Group size:	24 per sex and dose group
Test substance:	4-amino-2-hydroxytoluene
Code:	20010504
Purity:	99.9 area% (HPLC at 254 nm)
Dose levels:	0, 40, 200 and 1000 mg/kg bw/day
Route:	Oral, gavage
Vehicle:	1 % aqueous carboxymethylcellulose (CMC)
Dosing schedule:	Males: Once daily
	Females: Once daily
GLP:	In compliance

4-amino-2-hydroxytoluene was administered once daily by oral gavage to groups of 24 male and 24 female Wistar Crl:(WI) BR rats during the pre-mating (70 days for males, 14 days for females), mating, gestation and lactation period at doses of 40, 200 and 1000 mg/kg bw/day. A concurrent control group received the vehicle (1% aqueous carboxymethylcellulose) only. Animals were paired one/one for a maximally period of 21 days. Litters were raised until day 21 post partum.
Animals were observed once daily for clinical signs and twice daily for mortality during the entire treatment period. Body weights were recorded on the first day of exposure and weekly thereafter until necropsy. Food consumption was measured at weekly intervals until delivery (except during mating). During lactation, food consumption was recorded on days 1, 4, 7, 14 and 21 post partum.
Females without litters or which lost their litters were killed and necropsied. The sex ratio of pups was recorded at day 0, 4, and day 21 of lactation. Pup weights were noted on days 1, 4, 7, 14 and 21 of lactation. Pups were also observed daily for survival as well as for physical and behavioural abnormalities. The number of pups was reduced to 4 males and 4 females per litter on day 4 post partum. The remaining pups, from all litters with more than 4 pups per sex, were examined macroscopically. On day 21 post partum, all pups were examined internally and externally for abnormalities and, if indicated, skeletal development was examined after staining.
At necropsy of the parent animals, a macroscopical examination with special focus on the organs of the reproductive system was performed and several organs were weighed. The
histopathological investigation covered all gross lesions, cervix, coagulation gland, testes with epididymides, kidney, liver, ovaries, pituitary gland, prostate gland, seminal vesicles, uterus, vagina and target organs.

Results
Stability, homogeneity and content of the solutions of 4-amino-2-hydroxytoluene in the vehicle (1% CMC) were analytically confirmed. Dosing solutions were prepared daily within 4 hours prior to dosing and homogenised to a visually acceptable level. Homogeneity: determinations for the three test solutions revealed values of 95-102%, 96-107% and 94-107% related to the nominal value. The correctness of the dosing solutions was analytically verified at three time

points throughout the study. Low, mid and high dose values were in the range of 90- 97%, 98-109% and 85-112%, respectively.
No treatment-related findings were noted at 40 mg/kg bw/day.
In the 200 mg/kg bw/day group, all males and females showed brown discolouration of the urine during treatment. but no other treatment-related findings were noted.
At 1000 mg/kg bw/day, an increased incidence in mortality (2/24 males (about 8 %) and 5/24 females (about 21%)) was noted. Treatment-related clinical signs in dosed animals consisted of tonic spasms, lateral recumbency, rales, gasping, emaciation, hypothermia, lethargy, laboured respiration, pale appearance, chromodacryorrhoea and ptosis.
All males and females showed brown urine during treatment. Several body parts of both sexes showed brownish or yellow discolouration. Due to the staining properties of the test substance, these observations were not considered to be toxicologically relevant.
In males, decreased food consumption during pre-mating and decreased body weights during the complete treatment period were observed. In females, decreased food consumption occurred during the complete treatment period and decreased body weight gain during lactation.
In males, increased absolute and relative liver weights as well as increased relative weights of kidneys and testes were noted. During the histopathological examination, minor grades of hepatocellular vacuolation were found in females. Males showed increased incidence and severity (primarily slight or moderate) of midzonal/centrilobular liver hypertrophy and increased severity of renal corticomedullary tubular atrophy and hyaline casts. Reproduction parameters like slightly increased mean duration of gestation, delivery difficulties, and a decreased number of pups at birth were observed.
In this high dose group, developmental toxicity consisted of an increased post-natal loss resulting in a decreased viability index, as well as an increased incidence of clinical signs and decreased body weights in the offspring.

Conclusion
In this study, effects on reproduction, breeding and development were observed only at a dose level where marked systemic toxicity in parental animals (evident as increased mortality rate, severe clinical signs and reduced body weight gain and feed consumption) was noted. No effects at all were noted at 200 mg/kg bw/day or at a lower dose.
A clear NOAEL of 200 mg/kg bw for reproductive effects was deduced from this study.

Ref. 32

Comment
In males of the 200 mg/kg bw/day group a statistically significant increase in liver weight was noted, accompanied by microscopic changes (slight midzonal/centrilobular hypertrophy). In this group also relative kidney weight was increased. In the kidneys of male rats cortical hyaline droplets were recorded at an increased incidence and severity (also observed in the 1000 mg/kg bw/day group). In the 1000 mg/kg bw group an increase in severity of corticomedullary tubular atropy and hyaline casts were observed. In the 200 mg/kg group hyaline casts were more in evidence than in control animals. There were no treatment related alterations in the kidneys of female rats. Based on the morphology of the liver and kidneys, the NOAEL is 40 mg/kg bw/day for males and 200 mg/kg bw/day for females.
The above conclusion is in line with that from the pathology report included in the dossier. However, in the conclusion of the dossier, the effects on liver are not mentioned anymore, and the effects on the kidney are considered not of relevance for humans.

3.3.8.2. Teratogenicity

Guideline:	/
Species:	Rat, Sprague Dawley
Route:	oral by gavage
Group sizes:	25 mated females per dose group
Substance:	4-amino-2-hydroxytoluol
Batch:	2155/19/11
Purity:	/
Dose:	0, 20, 60, 180 mg/kg bw/day in volume of 10 ml/kg day
Exposure:	Gestation Days (GD) 6 -15
Recovery:	GD 16 -20
Vehicle:	1% carboxymethylcellulose (CMC)
Control groups:	Vehicle control
Positive control:	Vitamin A at 15 mg/kg bw/day (in rape oil)
GLP:	/ (unsigned QA statement included)

Mature male and female Sprague-Dawley rats were housed together, and day 0 of pregnancy was defined as the day on which vaginal sperm were detected. Females were then housed separately and dosed by gavage during the embryonic period GD 6 -15. Treated rats were observed daily and weighed on GD 0, 6, 15 and 20. On GD 20, dams were euthanized with CO_2, the uterine horns were removed and numbers and conditions of foetuses recorded Half the foetuses were fixed in Bouin's solution for examination of soft tissue, and the other half were fixed in ethanol and stained with Alizarin Red S for examination of skeletal effects.

Results

Stability and homogeneity data are not provided in the study. However, the test solutions of 4-amino-2-hydroxytoluene were prepared freshly immediately before administration. Consequently, it can be assumed that the test item solutions were homogenous and stable for the required single application. This assumption is further supported by findings in other studies with CMC-water solutions, in which a sufficient homogeneity and stability was analytically verified (see 4.8.1).

No treatment-related effects in dams were noted with regard to clinical observations and *post mortem* findings. The body weight gain in all treatment groups was comparable to that of the control group. Reproduction data such as pregnancy incidence, implantation rate or pre- and post implantation loss revealed no differences between treated and control groups. At gross necropsy no treatment-related effects were observed. The uterus and placenta weights, the number of corpora lutea, and implantation sites were similar to controls in all treated groups. There were no treatment-related effects with regard to litter size, foetal mortality, foetal body weight and sex ratio. The skeletal and visceral examination of the foetuses revealed no treatment-related findings. Neither a statistically significant difference as compared to the concurrent control, nor a dose-dependent increase in any malformation was noted. The observed variations represented common findings for the rat strain used and were within the spontaneous variation range and/or revealed no dose-response relation. Vitamin A produced marked teratogenic effects with exencephaly being the most pronounced malformation in the foetuses.

Conclusion

In this rat teratogenicity study, no treatment-related changes were noted in any of the dose groups of 20, 40 and 180 mg /kg bw/day administered from day 6 to day 15 post coitum.

Consequently, a NOAEL of 180 mg/kg bw for maternal and embryo-foetal effects is determined for 4-amino-2-hydroxytoluene

Ref: 33

Comment
For an adequate hazard assessment dose levels were too low (no rationale was given for dose selection)

3.3.9. Toxicokinetics

3.3.9.1. Toxicokinetics *in vitro*

Biovailability across intestinal barrier in TC-7 (human intestinal epithelial) cells

Guideline:	Not indicated
Cells:	Human intestinal epithelial cell line TC-7
Test substance:	4-Amino-2-hydroxytoluene
Batch:	20000506
Purity:	99.9 area% (HPLC, 254 nm)
Test concentration:	50 µM in HBSS buffer containing 1 % DMSO
Incubation time:	60 min
Number of experience:	Two independent experiments
GLP:	Not in compliance, but QAU checked

The bioavailability of 4-Amino-2-hydroxytoluene across the intestinal barrier was investigated in human intestinal epithelial (TC-7) cells *in vitro*. The permeability from the apical (A, pH 6.5) to the basolateral (B, pH 7.4) side was investigated at 37°C in 96-well transwell plates with shaking for a 60 min contact time. Analysis of the donor (apical) and receiver (basolateral) samples was done by means of HLPC-MS/MS and the apparent permeability coefficient (Papp) was calculated for two independent experiments. 14C-mannitol (about 4 µM) was used to demonstrate the integrity of the cell monolayer. Only monolayers revealing a permeability of < 2.5 x 10-6 cm/sec are used. Atenolol, propranolol, vinblastine and ranitidine were analysed concurrently to demonstrate the validity of the test system. According to the laboratories classification system, a low permeability is considered for test items revealing a Papp < 2 x 10-6 cm/sec. A Papp of 2 - 20 x 10-6 cm/sec and a Papp ≥ 20 x 10-6 cm/sec classify a substance to have a moderate and a high permeability, respectively. Ranitidine, which has a 50% absorption in humans was used as low permeability reference compound, as recommended by FDA.

Results
The total recovery for the reference substances and 4-Amino-2-hydroxytoluene ranged from 71 (test compound) to 96% (reference substances).
The figures for the reference substance ranitidine (Papp = 0.4 x 10-6 cm/sec) was well within acceptance range for compounds of 0.2 – 2 x 10-6 cm/sec and demonstrates the validity of the assay. The figure for the reference substance propranolol (Papp = 47.8 x 10-6 cm/sec), a high permeability reference compound with 90% absorption in humans, slightly exceeds the acceptance range for compounds of 20 – 45 x 10-6 cm/sec, nevertheless demonstrating the validity of the assay.
4-Amino-2-hydroxytoluene revealed a Papp of 103.8 x 10-6 cm/sec and thus was classified to be of high permeability, indicating a nearly 100 % absorption from the gastro-intestinal tract.

Conclusion
With 4-Amino-2-hydroxytoluene a mean permeability in human intestinal epithelial (TC- 7) cells of 103.8 x 10-6 cm/sec was obtained, which classifies the test item to be of high permeability. As the absorption from the gastro-intestinal tract is likely to be permeability limited, the high permeability observed in this assay indicates a good absorption of 4-Amino-2-hydroxytoluene after oral administration.

Ref.: 34

N-acetylation in the human keratinocyte cell line HaCaT

Guideline:	Not indicated
Cells:	Human keratinocyte cell line HaCaT
Test substance:	4-Amino-2-hydroxytoluene Batch: 20000506
Purity:	99.9 area% (HPLC, 254 nm)
Test concentration:	0.25 to 25 µg/ml in DMEM supplemented with 10% fetal calf serum
Incubation time:	24 hours
Number of experience:	Two independent experiments
GLP:	not in compliance

To determine the capacity of human skin to N-acetylate 4-Amino-2-hydroxytoluene, the human keratinocyte cell line HaCaT was used. Cells (875,000 ± 120,000 cells/ml) were exposed to the test substance in a concentration range from 0.25 to 25 µg/ml. After 24 hours the incubation was terminated and the formation of the N-acetylated metabolite 4-Acetylamino-2-hydroxytoluene as well as the remaining concentration of the substrate 4-Amino-2-hydroxytoluene was determined in supernatants after extraction and concentration before HPLC-DAD analysis. As a positive control substrate, the acetylation efficiency of paminobenzoic acid (PABA) was determined accordingly.

Results
The maximum substrate concentration leading to nearly complete N-acetylation was found to be in the range of 0.25 to 1 µg/ml. Additional experiments were performed in the low range of substrate concentrations (0.25-2.5 µg/ml) to analyze the optimum substrate concentration. After 24h, the lowest concentration of remaining substrate was observed at 0.25 µg/cm2, where 66% were recovered as acetylated metabolite.

With increasing substrate concentrations (up to 10 µg/ml), the concentrations of the acetylated metabolite increased linearly, but increasing concentrations of unmodified substrate were also detected, indicating a saturation kinetics of the responsible enzyme N-acetyltransferase 1 (NAT 1). At substrate concentrations above 10 µg/ml, cell viability and metabolic activity were impaired. As a positive control for the N-acetylation capacity of HaCaT cells, the well known NAT-1 substrate p-aminobenzoic acid (PABA) was used. At low concentrations (up to 10 µg/ml), HaCaT cells revealed a comparable N-acetylation capacity for PABA and 4-Amino-2-hydroxytoluene, indicating the validity of the test system.
The total recovery in the supernatant was incomplete, since approximately 30 % of the test substance could not be recovered probably due to unspecific modifications such as degradation and/or oxidation.

Conclusion

In conclusion, the capacity of human keratinocytes to N-acetylate 4-Amino-2-hydroxytoluene was found. At concentrations below 2.5 µg/ml the N-acetylation of 4-Amino-2-hydroxytoluene was almost complete. The validity of the test system was demonstrated by its capacity to N-acetylate the positive control substrate PABA.

Ref.: 38

Comparative metabolism study in primary hepatocytes of human, rat and mouse

Guideline:	None
Cells:	Hepatocytes from male humans (pooled from 3 donors) Hepatocytes from male Sprague Dawley rats Hepatocytes from male ICR/CD-1 mice
Cell density:	0.88 to 1.2 x 106 cells
Test substance:	4-Amino-2-hydroxytoluene
Batch:	20000506
Purity:	99.9 area% (HPLC)
Test concentration:	10 µM
Incubation time:	4 hours
GLP:	in compliance

The metabolic profile of 4-Amino-2-hydroxytoluene was investigated *in vitro* by means of cryo-preserved primary hepatocytes each from male human donors, male Sprague Dawley rats and male ICR/CD-1 mice.
The metabolic capacity of the used hepatocytes was characterised by marker substrates for phase I (CYP 2A6, 1A, 2A and 2B, 1A1/2 and 2E1) and phase II enzymes (N-Acetyl-transferase 1/2) used routinely or considered to be relevant for the metabolism of the class of arylamines, including 4-Amino-2-hydroxytoluene.
10 µM of the test item were incubated with approximately 1 x 10^6 cells/ml for a period of 4 h.
Samples were removed and analysed at 0, 0.5, 1.5 and 4 hours. Final test conditions were chosen based on range finding experiments.
Incubation was performed in 24-well microtiter plates and cell conditions were microscopically evaluated at each time point.
The metabolic stability was assessed by detection of loss of parent compound by means of LCMS/ MS. The metabolic profile was also investigated by LC-MS and metabolites identified/characterised as far as possible.

Results
Cell viability was not affected by the test item for the incubation period. A slight decrease in viability of about 10 % was noted at the end of the entire incubation period.
The marker substrates demonstrated the metabolic capacity and the validity of the test system.
As expected, differences in the metabolic capacity between rat, mouse and human hepatocytes were noted for the different phase I marker reactions. For phase II enzymes (N-acetyltransferase, NAT), liver samples each from humans (pooled in order to result in a intermediate metaboliser phenotype), rats (rapid metaboliser) and mice (mixed genotype of slow and rapid metabolisers) showed similar activities for the conversion of the model substrate *para*-amino benzoic acid, whereas with sulfamethazine (NAT 2) a much higher activity was noted for human than for rat and mouse hepatocytes.
4-Amino-2-hydroxytoluene was rapidly metabolised in human, rat and mouse hepatocytes. A decrease of 92.9%, 74% and 54.1% of the parent compound was detected within 1.5 h incubation

for human, rat and mouse hepatocytes, respectively. After 4 h, 4-Amino-2-hydroxytoluene was completely metabolised by both human and rat hepatocytes, in mice hepatocytes, 82% of the parent compound were converted. During the first 1.5 h, metabolism was linear with incubation time.
These data suggest that the overall metabolism rate in rodent and human hepatocytes is comparable. The analysis of the formed metabolites revealed an extensive phase II metabolic activity with sulfation of the phenol group of 4-Amino-2-hydroxytoluene predominating in hepatocytes from all three species.

Conclusion
Data obtained following exposure of rodent (mice, rats) and human hepatocytes to 4-Amino-2-hydroxytoluene under identical test conditions suggest no significant differences in the metabolic rate/capacity or the metabolic profile. Therefore, the results of this comparative *in vitro* metabolism study in hepatocytes support the validity of an extrapolation from rodent (rat) data to the human situation for phase II metabolism.

Ref.: 39

Hepatic metabolism: *in vitro* analysis in human, rat, and mouse

Guideline:	Not indicated
Test System:	Hepatocytes from humans (pooled from 4 female donors), female rats, and female mice (In Vitro Technologies, Inc) Pooled human, rat, and mouse liver microsomes (Gentest, Woburn, MA, USA)
Test substance:	^{14}C-4-Amino-2-hydroxytoluene 4-amino-2-hydroxytoluene (non-radiolabelled)
Batch:	CFQ14292 Batch 1 (Amersham Biosciences) - 5-amino-2-methyl[U-^{14}C]phenol Lot No. 203, Jos. H. Lowenstein and Sons, Inc.– non-radiolabelled *4*-4-amino-2-hydroxytoluene
Purity:	Radiochemical purity: 98.4% Non-radiolabelled 4-amino-2-hydroxytoluene: 99.9% (HPLC)
Test concentrations:	10 µM and 100 µM
Positive Control:	2-Aminofluorene
Negative Control:	p-Phenylenediamine
Incubation time:	Hepatocytes: 4 h
Hepatic microsomes:	60 min
GLP:	not in compliance

4-amino-2-hydroxytoluene, either radiolabelled or non-radiolabelled was incubated with pooled human, rat and mouse liver microsomal preparations for 60 min in the presence or absence of an NADPH-regenerating system. The metabolic capacity of the microsomal preparations was documented by the supplier. 2-Aminofluorene and p-phenylenediamine were used as reference controls in the human liver microsomal preparations. Reactions were stopped by the addition of acetonitrile. The samples were centrifuged and aliquots of the supernatants were analysed by HPLC-MS for the detection of parent compound and metabolites. Potential for binding to microsomal protein was determined by liquid scintillation counting of repeatedly washed microsomal pellets from reactions with the radiolabelled test material conducted in the presence or absence of an NADPH generating system.

Cryopreserved human, rat or mouse hepatocytes were pooled and incubated as a cell suspension ($1X10^6$ viable cells/ml) for 4 h with 4-amino-2-hydroxytoluene, either radiolabelled or non-radiolabelled. Incubations were stopped by the addition of acetonitrile, samples were centrifuged, and supernatants were analysed by HPLC-MS or by HPLC with radiochemical detection. For MS detection, total ion scanning in positive and negative mode was performed.
Subsequently single ion recording was carried out for specific metabolites of interest in order to detect parent compound and mono-oxygenated, di-oxygenated, mono-acetylated, diacetylated, sulfated, an glucuronidated metabolites.

Results
After incubation of ^{14}C-4-amino-2-hydroxytoluene with rat and human hepatocytes, two distinct mono-sulfated metabolites were detected. There was also evidence for the formation of an acetylated metabolite, especially in rat hepatocytes. Comparison with appropriate standards indicates that the mono-N-acetylated metabolite and the mono-N-sulfated metabolite were formed. The identities of a second sulfated metabolite (both rat and human) and a second acetylated metabolite (human) have yet to be confirmed. The sulfated metabolites were the most abundant metabolites in both rat and human. No mono-oxygenated metabolites or glucuronide metabolites were detected by LC followed by radiochemical detection. Considering the radiochemical detection limit, if such metabolites were present they would represent less than 5% of the total metabolites.
In experiments with LC/MS detection, 4-amino-2-hydroxytoluene was shown to be metabolised by rat, mouse and human hepatocytes to form one (rat and mouse) or two (human) mono-acetylated, one (rat) or two (human and mouse) mono-sulfated and one (rat) or two (human and mouse) glucuronide metabolites. Under the conditions used, the LC/MS detection method did not permit quantification. However, the LC/MS analysis, if regarded as semi quantitative, suggests that the glucuronide metabolites were present at less than 1%. This was consistent with the results of the radiochemical detection experiment where these metabolites were not found.
In human, rat and mouse liver microsomes incubated with 4-amino-2-hydroxytoluene in the presence of NADPH, two mono-oxygenated metabolites of 4-amino-2-hydroxytoluene were detected by LC/MS. Comparison with an appropriate standard indicated that one of these was the hydroxyl-methyl metabolite. Although the other metabolite was not identified, there was no evidence for enzymatically-mediated NADPH dependent covalent binding of 4-amino-2-hydroxytoluene to microsomal proteins, suggesting that the mono-oxygenated metabolites that were formed were not biologically reactive.

Conclusion
4-amino-2-hydroxytoluene is extensively metabolised via sulfation and, to a lesser extent, via N-acetylation by intact hepatocytes in all three species. The findings indicate that the hepatic metabolism of 4-amino-2-hydroxytoluene is similar in rodents and humans and includes predominantly Phase II conjugation (sulfation and N-acetylation).

3.3.9.2. Toxicokinetics *in vivo*

Absorption, distribution, metabolism and excretion of ^{14}C-*4-Amino-2-hydroxytoluene* in the Wistar rat

Guideline:	OECD 417 (1984)
Species/strain:	Rat, Wistar Kyoto, WKY/NR Crl BR (inbred)
Group size:	4 Females in the mass balance groups (groups 1, 2, 3, 4, 5) 6 Females in the toxicokinetic groups (groups 6, 7, 8, 9, 10)
Test substance:	*4-AMINO-2-HYDROXYTOLUENE*
Vehicle:	Intravenous administration: Ethanol:Alkamuls:Milli-Q 1:2.5:6.5
Oral administration:	Ethanol:Alkamuls:Milli-Q 1:5:4
Dermal administration:	Ethanol
Batch:	1 [ring-^{14}C(U)]-*4-Amino-2-Hydroxytoluene:* CFQ14292 Non-radiolabelled *4-Amino-2-Hydroxytoluene:* 20000506
Purity:	Radiochemical purity: 98.2% HPLC Non-labelled: 99.9 area% (HPLC, 254 nm)
Dose levels:	Intravenous administration: 12.5 mg/kg bw (containing approximately 0.8 MBq/mg of radioactivity) Oral administration: 12.5 / 500 mg/kg bw (containing approximately 0.8 / 0.02 MBq/mg radioactivity) Dermal administration: 15 / 45 mg/ml containing approximately 1 / 0.33 MBq/ml radioactivity (equal to 12.5 / 37.5 mg/kg bw and 0.15 / 0.45 mg/cm² skin)
Dose volume:	Intravenous administration: 2 ml/kg Oral administration: 10 ml/kg Dermal administration: 0.2 ml/animal
Route:	Intravenous, oral (gavage), dermal
Dosing schedule:	Oral: Single administration Dermal: Single application for 24 h, occlusive
GLP:	in compliance

Ten groups of rats were used in the study: five groups (n=4) for the mass balance and five groups (n=6) for the toxicokinetics. Rats were dosed intravenously with 12.5 mg/kg bw, orally with 12.5 mg/kg bw or 500 mg/kg bw and dermally with 15 and 45 mg/ml (equivalent to 12.5 and 37.5 mg/kg bw and to 0.15 and 0.45 mg/cm^2, respectively). For animals dosed by dermal application, ingestion of the test substance via grooming was prevented by collaring the animals.
Animals in the mass-balance groups were housed in metabolism cages in order to obtain a total ^{14}C-radioactivity material balance. Urine and faeces were collected in 0-6, 6-12, 12-24, 24-48, 48- 72 and 72-96 h intervals. Animals were euthanised 96 hours after dose administration, and several tissues and organs were collected. Total radioactivity in urine, faeces, tissues and organs was determined. For metabolism evaluation, selected urine and faeces samples were pooled per group and the metabolite profile in these pooled samples was investigated by TLC and LC-MS-MS.
In the toxicokinetic groups, blood was sampled alternately from two rats per time point at 0.25, 0.5, 1, 4, 8 and 24 h after dosing. Total radioactivity and 4-amino-2-hydroxytoluene equivalent concentrations were determined. Remaining plasma was pooled per group and the metabolite profile in these pooled samples was also investigated by TLC and LC-MS-MS.

Results
Homogeneity and stability of test substance in the vehicle were demonstrated by HPLC.

No mortality was observed in the study. Lethargy, piloerection and quick breathing were observed in one female of the high oral dose group. In the dermal groups a red discharge from the nose and eye was observed, due to the collar around the neck preventing the animals from grooming.
Oral absorption was calculated using the urine data (mass balance groups) and using the plasma data (toxicokinetic groups).
With the urine data, the absorption was calculated by dividing the percentage of radioactivity recovered in the urine after oral administration by the percentage of radioactivity recovered in the urine after intravenous administration. Calculation of the oral absorption from the urine data assumes that the ratio of urinary excreted radioactivity to systemically available radioactivity is constant for both oral and intravenous routes. The oral absorption calculated from the urine data was 95 % for the low dose group and 84% for the high dose group.
With the toxicokinetic (plasma) data, oral absorption was calculated by dividing the dose-normalized area under the curve (AUC) after oral administration by the AUC after intravenous administration. Calculated this way on a dose normalized basis, the oral absorption was 40 % for the low dose group and 38 % for the high dose group.
Furthermore, it was evident that oral absorption was fast, with T_{max} values of 0.5 h. The dose-normalized C_{max} value of the high oral dose group was much lower than the dose-normalized C_{max} of the low oral dose group. However, dose-normalized AUC values were similar between the low and high oral dose, indicating no changes in clearance with dose (e.g. no induction or saturation of elimination pathways). T_{max} values were also similar between the low and high dose, indicating no changes in the rate of absorption at the high dose. Therefore, a precise explanation for the lower dose-normalized C_{max} values is difficult, but might be due to a change in distribution volume.
The average dermal absorption calculated from the mass balance data was quite high (as intended by using ethanol as a vehicle), 53%, or 0.103 mg/cm^2 in group 4 (low dermal dose) and 32 % or 0.173 mg/cm^2 in group 5 (high dermal dose). If, in addition, the skin residue is considered as potentially systemically available to yield the "total potentially absorbed fraction" the values are 59 % of the applied dose, or 0.113 mg/cm^2 in group 4, and 35 % of the applied dose, or 0.193 mg/cm^2 in group 5, respectively.
When calculated with the toxicokinetic (plasma) data on a dose-normalized basis, the absorption was 7 % and 4 % in the low and high dose group, respectively. Furthermore, these data indicate that dermal absorption also proceeded rapidly, with a T_{max} of 0.25 or 0.5 h. Dose-normalized C_{max}, AUC and Fabs were 1.5 - 2 times lower for the high dermal dose group (group 10) compared to the low dermal dose group (group 9).
The difference in the absorption rate between the low and high dermal dose groups is likely to be due to saturation processes (e.g. saturation of metabolic enzymes) in the skin at the high dose level.

Urine was the most important route of excretion of 4-amino-2-hydroxytoluene independent of the route of exposure. Urinary excretion accounted for 94% of the applied dose after intravenous dosing, 89% after low oral dosing, 79% after high oral dosing, 39% after low dermal dosing and 18% after high dermal dosing. The pattern of urinary excretion after oral dosing (no saturation of elimination processes after high oral dosing) as well as after dermal dosing (saturation of absorption processes after high dermal dosing) confirmed the above described findings from the toxicokinetic data.

The rate of urinary excretion during the first 24 hours was different for the different groups, with the highest excretion in the first 6 hours for the intravenous and low oral dose group. For the high oral dose group and the dermal groups, excretion was more evenly divided over the 0-6, 6-12 and 12-24 hour intervals. However, after 24 hours the rate of excretion was similar for all groups (reflecting similar terminal half-lives of excretion for all routes of administration).
Excretion via faeces was only a minor route of elimination after intravenous, oral and dermal administration. Faecal excretion accounted for 6% after intravenous dosing, 11% after low oral dosing, 9% after high oral dosing, 4% after low dermal dosing and 2% after high dermal dosing.
Blood concentrations in group 1 (iv group) and 3 (high oral dose group) were 2.2 to 2.8 times higher than plasma concentrations, indicating a different distribution of the test substance (into the red blood cells), compared to groups 2 (low oral dose), 4 (low dermal dose), and 5 (high dermal dose), where plasma concentrations were below or at the limit of quantification.
At termination of the study, the average total remaining radioactivity in blood, carcass plus tissues was between 0.4 and 0.9% of the administered dose in the iv and oral groups, indicating no major accumulation of radioactivity after 96 hours. In the dermal groups, the total remaining radioactivity in blood, carcass plus tissues was 14.6 and 13.9% of the administered dose, in groups 4 and 5, respectively, including the treated skin.
The average total recovery of radioactivity in the mass balance groups (1 to 5) was between 89% and 103% of the applied dose.

The results of the mass balance and the toxicokinetic calculations are summarised in table 1 below:

Table 1

Group n°	**4-amino-2-hydroxy[U-^{14}C]toluene Dosage Level / Concentration**	Dosing route	Absorption (%)	Excretion via urine (%)	Excretion via Faeces (%)
Mass balance data					
1	12.5 mg/kg bw	iv	100	94	6
2	12.5 mg/kg bw	oral	95	89	11
3	500 mg/kg bw	oral	84	79	9
4	15 mg/ml; 12.5 mg/kg bw; 0.15 mg/cm²	dermal	53*	39	4
5	45 mg/ml; 37.5 mg/kg bw; 0.45 mg/cm²	dermal	32*	18	2
Toxicokinetic (plasma) data					
			F_{abs} (%)	C_{max} (mg/kg)	AUC∞ (h*mg/kg)
6	12.5 mg/kg bw	iv	n.a.	38.7	60
7	12.5 mg/kg bw	oral	40	11.6	24
8	500 mg/kg bw	oral	38	85.2	863
9	15 mg/ml; 12.5 mg/kg bw; 0.15 mg/cm²	dermal	6.6	1.3	6
10	45 mg/ml; 37.5 mg/kg bw; 0.45 mg/cm²	dermal	4.3	1.6	11

* without skin residue
F_{abs}: Absolute oral/dermal bioavailability, calculated as ((AUC0-∞ po or dermal/AUC0-∞ iv) * (dose iv/dose po or dermal)* 100%

Metabolite identification

Three major metabolites were detected in the urine and identified as the glucuronide, sulfate and N-acetyl (or N-acetylated and-sulfated metabolite) of the parent compound 4-amino-2-hydroxytoluene , respectively, using appropriate standards.
After dermal exposure, the major metabolite (66% of recovered radioactivity) is the N-acetylated (and/or N-acetylated and –sulfated) metabolite, and 14% of the radioactivity is either recovered as sulfate or glucuronide, leading to approx. 94% of the recovered urine metabolites. After oral exposure, the majority (51% of recovered radioactivity) is metabolised to sulfate and 32% of the radioactivity is identified as N-acetylated (and/or N-acetylated and – sulfated) metabolite and 10% as glucuronide, leading to approx. 93 % of the recovered urine metabolites.
In addition, the interpretation of the analysis for one minor metabolite peak (< 5%) in the urine of the high oral dose group indicated that it may originate from oxidation by phase I enzymes, but a precise quantification of the metabolites in this dose group was impossible due to a large concentration of unlabelled test substance in the sample.
The results on differences in urine metabolite formation between the different dose levels and routes of exposure are summarised in table 2 below.

Table 2: Individual contributions of the metabolites identified in urine samples of Wistar rats exposed to 4-Amino-2-hydroxy-[U-^{14}C]-toluene in % to the total metabolite pattern

Metabolite	Retention time (min)	m/z	1 iv	2 oral low	3 oral high	4 dermal low	5 dermal high
O-Glucuronide	7.9	-298	15	10	nq [1]	14	12
Sulfate	10.6	-202	41	51	nq	14	14
N-Acetyl-o-Glucuronide	11.8	+359	*(4)* [2]	2	nq	(4)	(4)
Carboxylated -/N-acetyl	12.3	-194	-	-	nq	-	-
N-Acetyl	15.9	-164	37	32	nq	66	66
N-Acetyl-/sulfate	15.9	-244	37	32	nq	66	66
∑ metabolites			97	95	nq	98	96

1) nq = Not quantifiable. Radioactivity profile of pooled urine samples from group 3 was disturbed by the high amount of unlabelled compound present in the sample. Therefore, the individual contributions of the different radioactivity peaks to the total radioactivity could not be calculated.

2) *(value in italics)* = Estimation for minor peaks (< 5% of total radioactivity): Although a small amount of radioactivity seemed to be present at the retention times at which these metabolites

eluted, no peaks with *m/*z values corresponding to these metabolites were detected in the MS-data at these retention times.

No parent compound was detected either in urine or in faeces, apart from the faeces of the intravenous and high oral dose group, where a minor radioactivity peak indicated the presence of parent compound.
The individual contribution of the single plasma metabolites to the plasma metabolite profile could not be determined, since matrix effects impeded the accurate quantification of the radioactivity peaks. No major differences between urine and plasma metabolite profiles were found. When comparing the profiles of the minor plasma metabolite peaks among the different dose groups, the intravenous group, the high oral, and high dermal dose group contained a peak that may originate from oxidation by phase I enzymes (carboxylated- N-acetyl metabolite). This peak was absent in the low dermal dose group.

Conclusion
After oral administration, 4-amino-2-hydroxytoluene was extensively absorbed, readily distributed into all organs, extensively metabolised and excreted via the urine. Oral absorption was high, regardless of the dose (84 - 95% of the applied dose).
Occlusive administration for 24 h in ethanol resulted in high dermal absorption values with saturation phenomena observed for the high dose group (0.173 mg/cm^2).
Three major metabolites (glucuronide, sulfate and N-acetyl) were detected in the urine after all routes of administration. Following oral administration, the most abundant metabolic reaction was sulfation. Following dermal application, *N*-acetylation was the most important metabolic pathway. No parent compound was detected either in urine or in faeces, apart from the faeces of the intravenous and high oral dose group, where a minor radioactivity peak indicated the presence of parent compound.

Ref.: 41

3.3.10. Photo-induced toxicity

3.3.10.1. Phototoxicity / photoirritation and photosensitisation

No data submitted

3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity

No data submitted

3.3.11. Human data

No data submitted

3.3.12. Special investigations

No data submitted

3.3.13. Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

(4-amino-2-hydroxytoluene)
(Oxidative/permanent)

Maximum absorption through the skin	**A (µg/cm^2)**	**=**	**3.48 µg/cm^2**
Skin Area surface	**SAS (cm^2)**	**=**	**700 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**2.44 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.04 mg/kg**
No observed effect level (mg/kg) (Embryo-foetal development, rat)	**NOAEL**	**=**	**180 mg/kg**
Margin of Safety	**NOAEL / SED**	**=**	**4500**

3.3.14. Discussion

Physico-chemical specifications
4-amino-2-hydroxytoluene is used in oxidative hair dye formulations at a final concentration of 1.5%, after mixing with peroxide developer. Stability of 4-amino-2-hydroxytoluene in marketed products is not reported.

General toxicity
Under the experimental conditions of a 90-day oral toxicity study, the NOAEL was determined to be 180 mg/kg bw.
A NOAEL of 200 mg/kg bw for reproductive effects was deduced from a one generation reproduction study.
In a rat teratogenicity study, a NOAEL of 180 mg/kg bw for maternal and embryo-foetal effects was determined.

Toxicokinetics
After oral administration, 4-amino-2-hydroxytoluene was extensively absorbed, readily distributed into all organs, extensively metabolised and excreted via the urine. Oral absorption was high, regardless of the dose (84 - 95% of the applied dose).
Three major metabolites (glucuronide, sulfate and N-acetyl) were detected in the urine after all routes of administration. Following oral administration, the most abundant metabolic reaction was sulfation. Following dermal application, *N*-acetylation was the most important metabolic pathway. No parent compound was detected either in urine or in faeces, apart from the faeces of the intravenous and high oral dose group, where a minor radioactivity peak indicated the presence of parent compound.

Irritation, sensitisation

Under the described test conditions, a 2.5 % aqueous solution of 4-amino-2-hydroxy toluene showed no skin irritating potential and transient irritation to the conjunctivae. It is a moderate to strong skin sensitiser under the described test conditions.

Dermal absorption
Under the described test conditions, a maximum absorption of 3.48 μg/cm^2 was observed when 1.5mg cm^2 4-amino-2-hydroxytoluene was applied under oxidative conditions in a typical hair dye formulation.

Mutagenicity
4-amino-2-hydroxytoluene was not mutagenic in *Salmonella typhimurium*. However, it induced mutations in mouse lymphoma L5178Y cells *in vitro* (small colonies indicating clastogenicity), micronuclei in human lymphocytes *in vitro*, and DNA strand breaks in Chinese hamster V79 cells, without metabolic activation. *In vivo,* 4-amino-2-hydroxytoluene did not induce micronuclei in mouse bone marrow, or unscheduled DNA synthesis in rat hepatocytes. In an *in vitro* comet assay, the test substance was found positive; however, its major metabolite was found negative. However, primary genotoxicity of 4-amino-2-hydroxytoluene could not be excluded in rat liver, where the comet assay indicated an increase in DNA strand breakage.
On the basis of the available data, the substance has no relevant mutagenic potential *in vivo*.

Carcinogenicity
No data submitted

4. CONCLUSION

Based on the information provided, the SCCP is of the opinion that the use of 4-amino-2-hydroxytoluene itself as an oxidative hair dye substance at a maximum concentration of 1.5% in the finished cosmetic product (after mixing with hydrogen peroxide) does not pose a risk to the health of the consumer, apart from its sensitising potential.

Studies on genotoxicity/mutagenicity in finished hair dye formulations should be undertaken following the relevant SCCNFP/SCCP opinions and in accordance with its Notes of Guidance.

5. MINORITY OPINION

Not applicable

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