EUROPEAN COMMISSION

Health & Consumer Protection
Directorate-General

Scientific Committee on Consumer Products

SCCP

OPINION ON

2,2'-Methylenebis-4-aminophenol HCl

COLIPA n° A155

Scientific Committees
on consumer products
on emerging and newly identified health risks
on health and environmental risks

The SCCP adopted this opinion at its 18th plenary of 16 December 2008

About the Scientific Committees

Three independent non-food Scientific Committees provide the Commission with the scientific advice it needs when preparing policy and proposals relating to consumer safety, public health and the environment. The Committees also draw the Commission's attention to the new or emerging problems which may pose an actual or potential threat.

They are: the Scientific Committee on Consumer Products (SCCP), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and are made up of external experts.

In addition, the Commission relies upon the work of the European Food Safety Authority (EFSA), the European Medicines Evaluation Agency (EMEA), the European Centre for Disease prevention and Control (ECDC) and the European Chemicals Agency (ECHA).

SCCP

Questions concerning the safety of consumer products (non-food products intended for the consumer).
In particular, the Committee addresses questions related to the safety and allergenic properties of cosmetic products and ingredients with respect to their impact on consumer health, toys, textiles, clothing, personal care products, domestic products such as detergents and consumer services such as tattooing.

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Opinion to be cited as: SCCP (Scientific Committee on Consumer Products), Opinion on 2,2'-methylenebis-4-aminophenol HCl, 16 December 2008

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1. BACKGROUND

Submission I on 2,2'-methylenebis-4-aminophenol HCl was submitted in March 2003 by COLIPA[1].

2,2'-Methylenebis-4-aminophenol HCl is used as a hair dye up to a final concentration of 2.0% on head in the presence or absence of a developer-mix.

2. TERMS OF REFERENCE

1. *Does SCCP consider 2,2'-methylenebis-4-aminophenol HCl safe for consumers when used as an ingredient in oxidative and non-oxidative hair dye products with a maximum concentration of 2.0% on the scalp, taken into account the scientific data provided?*

2. *Does the SCCP recommend any further restrictions with regard to the use of 2,2'-methylenebis-4-aminophenol HCl in oxidative and non-oxidative hair dye formulations?*

[1] COLIPA – The European Cosmetic and 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

2,2'-Methylenebis-4-aminophenol HCl (INCI)

3.1.1.2. Chemical names

Bis-(5-amino-2-hydroxyphenyl)-methane . 2 HCl

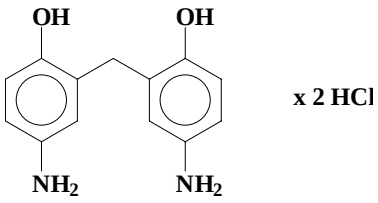
3.1.1.3. Trade names and abbreviations

Ro 1525 (SAT 980375, SAT 000344)
COLIPA n° A155

3.1.1.4. CAS / EINECS number

CAS: 27311-52-0 (dihydrochloride)
63969-46-0 (free base)
ELINCS: 440-850-3 (Ro 1525)

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_{13}H_{14}N_2O_2$. 2 HCl

3.1.2. Physical form

Light grey powder

3.1.3. Molecular weight

Molecular weight: 230.27 (free base)

3.1.4. Purity, composition and substance codes

Batch: Ro-RN 6567-083

Identification: NMR
NMR purity: > 97%

HPLC purity (peak area): 99.792%

The HPLC chromatogram provided in the summary is different from that included in the certificate of analysis (Ref. 15 Teratogenicity testing). The difference in the two HPLC chromatograms may be related to the different HPLC conditions used in the two cases.

3.1.5. Impurities / accompanying contaminants

At least three unidentified peaks in the HPLC chromatograms were observed.

3.1.6. Solubility

soluble in water, ethanol and methanol
pH 3.22 of 1% (w/v) solution in deionised water

3.1.7. Partition coefficient (Log P_{ow})

Log Po/w: /

3.1.8. Additional physical and chemical specifications

Melting point: /
Boiling point:
Flash point:
Vapour pressure:
Density:
Viscosity:
pKa:
Refractive index:
pH:

UV_Vis spectrum (200-800 nm): Two different UV spectra are provided for the same batch of Ro1525:
1) Absorption peaks at 212 nm (λmax), 232nm and 300nm (reported under physico chemical properties)
2) 208nm (λmax), 277nm and 300 nm (according to certificate of analysis provided with teratogenicity study (Ref. 15)

3.1.9. Homogeneity and Stability

Stable at room temperature in the dark (no data). Solutions should be prepared fresh daily.

General Comments to physico-chemical characterisation

- The UV spectrum of the test material used in the teratogenicity study is different from the UV-spectrum of same batch of 2,2'-Methylenebis-4-aminophenol HCl provided with its physico-chemical properties.
- Melting point of 2,2'-Methylenebis-4-aminophenol HCl is not reported
- Impurities in 2,2'-Methylenebis-4-aminophenol HCl have not been characterised
- Quantitative information on solubility of 2,2'-Methylenebis-4-aminophenol HCl in water and other solvents are not provided
- Log Pow of 2,2'-Methylenebis-4-aminophenol HCl is not provided

- No data is provided on the stability of Methylenebis-4-aminophenol HCl in the marketed products

3.2. Function and uses

2,2'-Methylenebis-4-aminophenol HCL is used as hair dye up to a final concentration of 2.0 % on head in the presence or absence of a developer-mix.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Guideline:	OECD 423 (1996)
Species/strain:	Sprague-Dawley rat
Group size:	3 males and 3 females
Test substance:	RO 1525
Batch:	Ro-Rn 6567-083
Purity:	not available
Dose:	0, 25, 200 and 2000 mg/kg bw; females 25 mg/kg bw only
Route:	Oral
Exposure:	single administration and 14 days observation
GLP:	in compliance
Study period:	12 May – 14 July 1998

Three female Sprague-Dawley rats were exposed to RO 1525 at the dose of 25 mg/kg. Nine male Sprague-Dawley rats were exposed to RO 1525 at the doses of 25, 200 and 2000 mg/kg, respectively. Aliquots of 20 ml/kg in water were administered orally by gavage. Animals were observed twice daily for mortality/morbidity and daily for clinical signs over a period of 14 days.

Results
At 2000 mg/kg all animals died within 1 hour after exposure. All animals exposed to 200 mg/kg died within 3 days. Sedation and unconsciousness were observed in both dose levels. Dyspnoea and tremor were observed in 200 mg/kg group. No mortality was observed at 25 mg/kg group, where clinical signs were piloerection, closed eyes, chromodacryorrhoea, pale skin, retention of faeces, discoloured urine and hunched posture. At necropsy exsiccosis, small spleen, gastric ulcers, gastric, intestinal and pulmonary haemorrhages, clear liquids in the thoracic cavity were noted in 200 mg/kg group. No differences between sexes were noted at 25 mg/kg.

Conclusions
The maximal non-lethal dose of RO 1525 was higher than 25 and lower than 200 mg/kg bw after a single oral administration in fasted rats.

Ref.: 1

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: OECD 404 (1992)
Species: New Zealand White rabbits
Group: 3 females
Substance: Ro 1525 (SAT 980375)
Batch: Ro-Rn 6567-083
Purity: 99.8% (HPLC)
Dose: 0.5 g of Ro 1525
Vehicle: /
GLP: in compliance
Study period: May 1998

Approximately 0.5 g of the test substance, spread over an area of approximately 6 cm² and moistened with 1.0 ml deionised water was applied semi-occlusive to the test site for 4 hours. The skin was examined at 1, 24, 48 and 72 hours after patch removal.

Results
No general toxic effects of the test substance were observed.
A grey staining of the skin at the application site was seen in all animals 1 and 24 h after patch removal. Except for very slight erythema, observed in 2/3 animals 1 h after patch removal, no lesions were noted at the other examination terms.

Time after patch removal	**Erythema / Eschar Animal number**			**Oedema Animal number**		
	21	**22**	**23**	**21**	**22**	**23**
1h	1	1	0	0	0	0
24h	0	0	0	0	0	0
48h	0	0	0	0	0	0
72h	0	0	0	0	0	0
Mean (24-72)	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**

Conclusion
The study authors concluded that the test substance "Ro 1525" did not cause any skin irritation or corrosion in this study.

Ref.: 2

Comment
The SCCP concluded that the neat test substance did cause mild and transient skin irritation.

3.3.2.2. Mucous membrane irritation

Guideline: OECD 405 (1987)
Species: New Zealand white rabbits
Group: 3 females
Substance: Ro1525
Batch: Ro-Rn 6567-083
Purity: 99.8% (HPLC)
Dose: 0.1 ml or 90 mg of undiluted test substance

Vehicle: /
GLP: in compliance
Study period: 12 May – 14 June 1998

The equivalent of 0.1 ml test substance was instilled into the conjuctival sac of one eye of each of 3 rabbits. The other eye remained untreated and served as negative control. The eyes were rinsed with warm water after 24h.

Results
The following mean scores were calculated from the individual examinations performed 24, 48 and 78h p.a.

Cornea	0	1	0
Iris	0	0.33	0
Conjunctivae, redness	2	3	2.67
Conjunctivae, chemosis	2.33	4	2.67

Ocular lesions were reversible in all animals. A high inter-individual variation in the response to the test substance was seen.

Conclusion
The study authors concluded that the test substance was irritant to the rabbit eye.

Ref.: 4

HET-CAM

Guideline: /
Species: Chorionallantoic Membrane (HET-CAM)
Substance: Ro 1525
Batch: Ro-Rn 6567-083
Purity: > 98%
Concentration: neat substance
45% aqueous suspension (300 µl)
8% aqueous suspension (300 µl)
Vehicle: water
Reference: Texapon ASV (5% active substance)
GLP: in compliance
Study period: 22 – 31 March 1998

The eye irritation potential of Ro 1525 was assessed using the Hen´s-Egg-Test (HET-CAM). The undiluted as well as the diluted (45% and 8% aqueous suspension) test substance was applied on six fertilised chicken eggs per concentration. The test substance remained in contact with the CAM for 30 seconds. Then, it was rinsed off with physiological saline solution.

Results
The neat test substance caused slight to moderate haemorrhage and lysis of vessels after 30 seconds of exposure. The 45% dilution caused only slight haemorrhage and lysis of vessels.
To better estimate the irritant potential of Ro 1525, an 8% dilution was tested using the "reaction time method".

Test Substance	**Relative irritation potential**	**Sum of individual scores**		**Conclusion**
Ro 1525	0.07 (8% AS)	8 (98% AS)	3 (45% AS)	Moderately irritant
Reference	1.00	12		Moderately irritant

Conclusion
The study authors concluded that the test substance was moderately irritant.

Ref.: 3

Comment
The HET-CAM test is a screening test, meant to screen out severe eye irritants for the purpose of labelling or classification of chemicals, but has not been officially validated as a stand alone replacement test for eye irritation. On its own it is not a suitable test for quantitative risk assessment of cosmetic ingredients. In this particular case it just confirms the previous in vivo results of a Draize eye test.

3.3.3. Skin sensitisation

Guinea Pig Maximisation Test

Guideline:	OECD 406	
Species:	Ibm:GOHI; SPF-quality guinea pig (Himalayan spotted)	
Group:	15 females (10 test and 5 control)	
Test substance:	Ro 1525	
Batch:	Ro-RN-6567-083	
Purity:	> 98% (HPLC)	
Doses:	0.1 ml	
Concentration:	intradermal induction:	10% in purified water and in 1:1 (v/v) mixture of Freund's complete adjuvant
	Epidermal induction:	25% in purified water
	Epidermal challenge:	3% in purified water
Vehicle:	purified water	
GLP:	in compliance	
Study period:	17 July – 26 August 2002	

In order to assess the cutaneous allergenic potential of Ro 1525, the Maximization-Test was performed in 15 (10 test and 5 control) female albino guinea pigs.
The intradermal induction of sensitization in the test group was performed in the nuchal region with a 10% dilution of the test item in purified water and in an emulsion of Freund's Complete Adjuvant (FCA) / physiological saline. The epidermal induction of sensitization was conducted for 48 hours under occlusion with the test item at 25% in purified water one week after the intradermal induction. The animals of the control group were intradermally induced with purified water and FCA/physiological saline and epidermally induced with purified water under occlusion.
Two weeks after epidermal induction the control and test animals were challenged by epidermal application of the test item at 3% in purified water and purified water alone under occlusive dressing. Cutaneous reactions were evaluated at 24 and 48 hours after removal of the dressing. To facilitate the reading by removing the black discoloration on the stratum corneum, the application area was stripped 4 to 5 times with Scotch Tape approximately 4 hours prior to the 24-hour reading.

Results
No toxic symptoms were evident in the guinea pigs of the control or test group. No deaths occurred. None of the control and test animals showed skin reactions after the challenge treatment with Ro 1525 at 3 % (w/w) in purified water.

Conclusion
The study authors concluded that the test substance was not a sensitiser.

Ref.: 5

Buehler Test

Guideline:	OECD 406 (1992)
Species:	Dunkin Hartley guinea pig, HsdPoc:DH
Group:	30 females (20 test, 10 control)
Test substance:	Ro 1525
Batch:	Ro-RN-6567-083
Purity:	/
Doses:	0.1 ml
Concentration:	1, 5, 25 and 50% Ro 1525 in white petrolatum (selection of test concentrations) Epicutaneous induction: 50% Ro 1525 in white petrolatum Epicutaneous challenge: 50% Ro 1525 in white petrolatum
Vehicle:	white petrolatum
Positive control:	α-hexylcinnamaldehyde
GLP:	in compliance
Study period:	12 May – 17 June 1998

Twenty female guinea pigs were used as a test substance group and another 10 females were used as a negative control group. There were three epicutaneous induction exposures and one epicutaneous challenge exposure. The concentration of the test substance was 50% in white petrolatum for all three induction exposures and for the challenge exposure. The areas of administration were covered occlusively for 6 hours.

Results
General
All animals survived till the end of the study. Possible skin reactions excluded, no other adverse effects were noted. The test substance did not stain the skin of the guinea pigs and scoring was not impeded by skin dyeing.

Skin reactions after induction exposures
The application sites of all control animals were normal at each time. In the test substance group, very slight to well defined erythema and/or oedema were noted in 3/20 animals after the second and/or third induction exposure.

Skin reactions after challenge exposure
In the negative control group, the control areas and also the test substance treated areas of all animals were normal 24 and 48 hours after the end of the challenge exposure.
In the test substance group, a well defined skin reaction was noted in 1/20 animals at the test substance treated area 24 hours and 48 hours after the end of the challenge exposure. Therefore one animal (5% of the test substance group animals) was regarded as sensitised.

Conclusion
The study authors concluded that the test substance was not a sensitiser.

Ref.: 6

Comment
As one animal was sensitised, the test substance should be considered as having some skin sensitising potential.

3.3.4. Dermal / percutaneous absorption

Guideline:	/
Tissue:	dermatomed pig skin, 2 donors (1 male and 1 female)
Group size:	6 replicates per experiment
Diffusion cells:	flow-through diffusion cell

Skin integrity:	transdermal electrical resistance (≥ 10 KΩ)
Test substance:	Ro 1525 ^{14}C-labelled Ro 1525 (95.3 µCi/mg)
Batch:	CFQ11562 (labelled)
Purity:	/ (non-labelled) 95.0% (radiochemical purity by HPLC)
Test item:	Formulation A: 1 part of the preparation (items 1 to 8) was mixed with 1 part of water for the experiments A1 and A2 Formulation B: 1 part of the preparation (items 1 to 8) was mixed with 1 part of developer for the experiments B1 and B2
Doses:	40 mg/cm² formulation (the actual dose was about 10% higher than the nominal dose of 40 mg/cm²)
Receptor fluid:	Dulbecco's phosphate buffered saline with 3% bovine serum albumin
Solubility receptor fluid:	/
Stability:	/
Method of Analysis:	liquid scintillation counter
GLP:	in compliance
Study period:	16 December 1999 – 18 February 2000

Composition of formulation

Item	Ingredient	Amount (mg)	Amount (%)
1	Ro 1525	27	3.6
2	^{14}C-labelled Ro 1525	3	0.4
3	Crème-Basis Bth 66	375	50.0
4	2-Amino-3-hydroxypyridine	10.8	1.44
5	Sodium sulphite	7.5	1
6	Ammonium sulphate	7.5	1
7	Ammonium, 25%	30	4
8	Water, dist.	289.2	38.56
Sum		750	100.00
9a	Water, for formulation A	750 µl	100
9b	Developer (containing H2O2), for formulation B	750 µl	100

The dermal absorption of Ro 1525 was studied as an ingredient of two representative formulations (A and B) on dermatomed skin preparations of two young pigs. For sensitivity reasons and to ensure a maximum recovery (mass balance) the test substance was [14C]-labelled.

Two independent experiments were performed with each formulation using six integrity checked skin preparations in each experiment. The experiments were performed in flow through penetration cells with an application area of 0.5 cm². The non-occlusive exposure under temperature controlled conditions lasted 30 minutes before rinsing.

The test substance formulations were applied topically to the horny layer of the skin in quantities of 22.9 mg (A) and 22.2 mg (B) respectively, which corresponded to 469 µg (A) and 453 µg (B) of the test substance. 48 hours after the application the stratum corneum was removed by repeated stripping with adhesive tapes to obtain the adsorbed test substance. The remaining skin was taken to determine the absorbed test substance. The penetration was calculated from the [14C]-amount in the fractionated receptor fluid consisting of phosphate buffered saline plus 3% bovine serum albumin. The overall amount of bioavailable test substance is defined as the sum of absorbed and penetrated quantities.

The means of test results are presented in the following table:

Parameter	Formulation A µg/cm²	Formulation A %	Formulation B µg/cm²	Formulation B %
Skin rinsings	/	89.5	/	85.2
Adsorption	31	3.3	24	2.6
Absorption	8.2	0.91	3.5	0.39
Penetration	1.0	0.11	0.085	0.009
Bioavailability	**9.2**	1.02	**3.6**	0.41
Mass balance	/	94.7	/	89.1

Individual results, experiment A1 (pig 1, female)

Skin sample	1	2	3	4	5	6	Mean (µg/cm²)
Absorption	19.40	6.17	3.39	12.89	10.69	12.62	
Penetration	0.29	0.20	2.51	0.71	1.32	3.68	
Bioavailability	**19.69**	**6.37**	**5.90**	**13.6**	**12.01**	**16.3**	**12.31 ± 5.45**

Individual results, experiment A2 (pig 2, male)

Skin sample	7	8	9	10	11	12	Mean (µg/cm²)
Absorption	12.15	0.26	13.36	0.98	0.59	5.44	
Penetration	0.42	0.20	0.07	0.69	1.69	0.62	
Bioavailability	**12.57**	**0.46**	**13.43**	**1.67**	**2.28**	**6.06**	**6.08 ± 5.68**

Individual results, experiment B1 (pig 1, female)

Skin sample	13	14	15	16	17	18	Mean (µg/cm²)
Absorption	8.92	6.39	5.07	0.13	1.06	7.82	
Penetration	0.12	0.04	0.05	0.06	0.39	0.06	
Bioavailability	**9.04**	**6.43**	**5.12**	**0.19**	**1.45**	**7.88**	**5.02 ± 3.53**

Individual results, experiment B2 (pig 2, male)

Skin sample	19	20	21	22	23	24	Mean (µg/cm²)
Absorption	1.91	2.28	1.07	1.14	3.85	2.02	
Penetration	0.02	0.01	0.11	0.05	0.10	0.01	
Bioavailability	**1.93**	**2.29**	**1.18**	**1.19**	**3.95**	**2.03**	**2.10 ± 1.02**

Conclusion
In the oxidative formulation, the amount considered absorbed was 3.56 (range 0.19 to 9.04) µg/cm² [0.41% of the applied dose]. Under non-oxidative conditions, the amount considered absorbed was 9.20 (range 0.46 to 19.69) µg/cm² [1.02% of the applied dose].
Ref.: 10

Comment
Too few replicates and an insufficient number of donors were used in this study. The amount of formulation applied (40 mg/cm²) was too high (normally 20 mg/cm²). An application area of only 0.5 cm² was used.
Under oxidative conditions, an A_{max} of 9.04 µg/cm² may be used for calculating the MOS. Under non-oxidative conditions, the A_{max} is 19.69 µg/cm² may be used for calculating the MOS.

3.3.5. Repeated dose toxicity

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

Guideline:	OECD 407
Species/strain:	Sprague-Dawley rat
Group size:	5 per sex
Test substance:	RO 1525
Batch:	Ro-Rn 6567-083
Purity:	> 98%
Dose:	0, 60, 80 and 100 mg/kg bw
Route:	gavage
Exposure:	once a day for 4 weeks
GLP:	in compliance
Study period:	21 April – 19 May 2000

The test substance, suspended in distilled water, was administered at 60, 80 and 100 mg/kg bw/day daily for 28 days by gavage. The control animals received vehicle only. All animals were observed twice daily for mortality and once daily for clinical signs. Blood samples were taken from all animals during week 4 for haematological and clinical chemistry investigations. At autopsy, organ weights were recorded and the main organs were examined macroscopically and histologically.

Results
No mortality occurred due to the test substance. There were neither treatment-related differences in body weights nor differences in haematology or clinical chemistry parameters between the exposed and control groups. The organs weight was comparable between exposed and control animals and no macroscopic changes of specific organs were observed. Brown pigmentation in the cell cytoplasm of proximal renal tubules and mild chronic interstitial inflammation, mainly localised in the cortical area, were observed in all treated animals of the high dose group. These lesions were seen, on most occasions, to be associated with tubular cell basophilia and tubular dilatation.
Moderate tubular cell basophilia associated with interstitial chronic inflammation was detected in males and females of the low and mid-dose group. In a number of males from the low-dose group, these findings were associated with vacuolation of cortical tubular cells. Brown pigmentation in the cell cytoplasm of the proximal renal tubules was again seen in animals of both sexes, particularly evident in males of the low and mid-dose groups.

Conclusions
Microscopic examination showed treatment-related changes in the kidney of animals from all treated groups (brown pigmentation in the cell cytoplasm of the proximal renal tubules) The authors reported a significant background incidence of renal changes (tubular cell basophilia associated with interstitial chronic inflammation) also in the control animals - typical to this strain of rats - which limits the evaluation of the toxicological significance of findings.

Ref.: 11

Comment
The SCCP considers the LOAEL to be 60 mg/kg bw.

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

Study 1

Guideline:	OECD 408
Species/strain:	Sprague-Dawley rat

Group size:	15 males and 15 females; additional 5 per sex in high dose satellite group
Test substance:	RO 1525
Batch:	Ro-Rn 6567-083
Purity:	> 98%
Dose:	0, 60, 80 and 100 mg/kg bw in water
Route:	by gavage
Exposure:	once a day for 13 weeks
GLP:	in compliance
Study period:	6 July – 6 November 2000

Three groups of 15 rats/sex received RO 1525 daily by oral gavage at doses of 0, 60, 80 and 100 mg/kg bw for 13 weeks. A control group received a vehicle only. Additionally 10 rats (5 per sex), for both the control and the high dose group, were assessed for recovery of treatment-related effects, four weeks after the last administration. Animals were observed twice daily for mortality/morbidity and once daily for clinical signs. Body weight and food consumption were determined before the exposure. Haematology, clinical chemistry and urinalysis evaluations were performed on week 13. Ophthalmologic evaluations of all animals in all groups were examined just prior to the exposure and re-examined during week 12 of treatment. All animals were subjected to a macroscopic examination and required tissues from animals in the control and exposed groups were evaluated microscopically.

Results
No deaths occurred during the course of the study. Detailed clinical signs with neurotoxicity assessment did not show any signs which could be clearly related to the treatment with the test item. Hyperaesthesia, tachypnoea and a slow arousal were occasionally noted. A slight statistically significant reduction in body weight gain was observed in the high dose females. However, this difference was no longer evident at the end of the recovery period. No findings attributed to treatment were seen at the ophthalmic examinations. A slight statistically significant decrease in haematocrit and haemoglobin were observed in the mid and high dose males on week 4. Mean corpuscular haemoglobin concentration was statistically significantly increased in high dose group. Several clinical chemistry and urinalysis parameters were affected in high dose animals. Statistically significant increase in relative kidney weight was seen in the mid and high dose males and in the high dose females. The change disappeared at the end of the recovery period. The histopathological evaluation of the kidneys in the control group and in all dose groups and in animals killed after the recovery period showed tubular degeneration. Tubular degeneration in the recovery female animals showed however a decreased incidence.

Conclusions
Renal effects in kidney are reported in the control group and at all dose levels investigated and therefore a NOAEL cannot be established.

Ref.:12

Study 2

Guideline:	OECD 408
Species/strain:	Wistar rat, Sprague-Dawley
Group size:	10 males and 10 females; additional 5 per sex in control and high dose satellite group (Wistar). Additional 5 male in control and high dose satellite groups (Sprague-Dawley)
Test substance:	RO 1525
Batch:	Ro-Rn 6567-083
Purity:	> 98%
Dose:	0, 5, 15 and 60 mg/kg bw in water

Route: by gavage
Exposure: once a day for 13 weeks
GLP: in compliance
Study period: 2 April – 30 July 2001

Three groups of 10 rats/sex received RO 1525 daily by oral gavage at doses of 5, 15 and 60 mg/kg bw in distilled water for 13 weeks. An additional satellite group was set up to control and high dose groups by using five Sprague-Dawley rats/groups. Animals were observed twice daily for mortality/morbidity and once daily for clinical signs. Body weight gain and food consumption were recorded. Haematology, clinical chemistry and urinalysis evaluations were performed on week 13. Ophthalmologic evaluations of all animals in all groups were examined just prior to the exposure and re-examined during week 12 of treatment. All animals were subjected to a macroscopic examination and required tissues from animals in the control and exposed groups were evaluated microscopically.

Results
One female from the low dose and two males from the high dose groups were found dead during the study. In addition, 1 control female and 2 males from the mid dose group died due to technical problems during the bleeding procedure. Detailed clinical signs with neurotoxicity assessment did not show any signs which could be clearly related to the treatment with the test item. Body weight gain and food consumption were not affected. No findings attributed to treatment were seen at the ophthalmic examinations. Haematological parameters were not affected in any exposed groups. In clinical chemistry parameters a statistically significant increase in aspartate aminotransferase, total protein and albumin was observed in high dose males. In urinalysis parameters an increased number of epithelial cells and leukocytes were noted in all exposed groups compared to controls at the end of exposure. Statistically significant increase in relative kidney weight was seen in the high dose males (in main and satellite groups). Relative spleen weights were increased in high dose group. This change disappeared at the end of the recovery period. The histopathological evaluation of the kidneys in mid and high dose groups and in animals killed after the recovery period showed tubular degeneration and pigmentation.

Conclusion
On the basis of these results, the study authors considered the No Observed Effect Level (NOEL) to be 5 mg/kg bw per day.

Ref.: 13

Comment
In study 1 and study 2, two distinct, apparently unrelated, patterns of pathological changes were observed in the kidneys. There was a clearly spontaneous set of changes (nephropathy) seen in both control and treated Sprague Dawley rats (study 1), but not in any Wistar rat (study 2). The most important feature of this nephropathy was cast formation or thickened basement membrane and tubular basophilia. This type of spontaneous pathology has long been recognised in the Sprague Dawley rat (ref. AD1). Based on the tubular degeneration in Wistar rats observed on the exposure level of 15 mg/kg bw /day but not at the level of 5 mg/kg/day, the NOAEL is set at 5 mg/kg/day.

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 Reverse Mutation Test

Guideline:	OECD 471
Species/strain:	TA 1535, TA 1537, TA 98, TA 100 and TA 102
Replicates:	Triplicates in two independent experiments
Test substance:	Ro 1525
Solvent:	Deionised water
Batch:	Ro-Rn 6567-083
Purity:	>98% by HPLC
Concentrations:	33, 100, 333, 1000, 2500, 5000 μg/plate
Treatment:	Experiment 1: Standard plate incorporation assay Experiment 2: Pre-incubation assay Both assays with and without Phenobarbital/β-Naphthoflavone induced rat liver S9-mix
GLP:	In compliance
Study period:	June 1998

To evaluate the toxicity of the test article a pre-experiment was performed with strains TA 98 and TA 100. Eight concentrations between 3 and 5000 μg/plate were tested in triplicates. The plates showed normal background growth up to 5000 μg/plate in both strains.

Results
Toxic effects, evident as a reduction in the number of revertants occurred at the following concentrations:

Strain	**Experiment I**		**Experiment II**	
	without S9-mix	**With S9-mix**	**without S9-mix**	**With S9-mix**
TA 1535	5000	no toxic effect	1000-5000	no toxic effect
TA 1537	1000-5000	2500	1000-5000	no toxic effect
TA 98	no toxic effect	no toxic effect	2500-5000	no toxic effect
TA 100	5000	no toxic effect	5000	no toxic effect
TA 102	no toxic effect	no toxic effect	No toxic effect	no toxic effect

There were no signs of an increase in the number of revertants in any of the five tester strains at any concentration tested neither with nor without metabolic activation.

Conclusion
Under the test conditions used Ro 1525 did not induce gene mutations in bacteria.

Ref.: 7

***In vitro* chromosome aberration test**

Guideline:	OECD Guideline 473
Species/strain:	Chinese hamster cells V79
Replicates:	Two replicates in one experiment
Test substance:	Ro 1525
Solvent:	deionised water
Batch:	Ro-Rn 6567-083

Purity:	> 98% (HPLC)
Concentrations:	Without metabolic activation: 2.5, 3.0, 3.5 and 4.0 µg/ml With metabolic activation: 15, 30 and 60 µg/ml
Treatment:	Chromosomes were prepared 18 hours after start of treatment. The treatment interval was four hours with and without metabolic activation (S9-mix induced by Phenobarbital/β-Naphthoflavone)
GLP:	In compliance
Study period:	15 June – 25 August 1998

A preliminary toxicity test was conducted using eight concentrations between 39.1 and 5000 µg/ml. Reduced cell numbers below 50% of control were observed after treatment with 312.5 µg/ml and above in the presence of metabolic activation and at concentrations of ≥ 39.1 µg/ml in the absence of metabolic activation. Precipitation of the test article in culture medium was observed after treatment with ≥ 156.3 µg/ml in the absence of metabolic activation and ≥ 2500 µg/ml in the presence of metabolic activation. At each test concentration two parallel cultures were used. Appropriate negative and positive controls were included.

Results
Without metabolic activation concentrations between 1.4 and 40 µg/ml were tested and strong toxicity was observed. Therefore, another test was performed with concentrations between 1-4 µg/ml. Using these concentrations, neither mitotic indices nor cell numbers were reduced to values below 50% of the control. The test article induced concentration related and significant increases in the number of cells carrying structural chromatid and chromosome aberrations.
In the presence of metabolic activation, reduced mitotic indices below 50% of the control were observed. There were no indications of an increase in the number of cells carrying structural chromatid and chromosome aberrations at the tested concentrations.
There were no indications of increases of polyploidy metaphases neither in the absence nor in the presence of metabolic activation.

Conclusion
The conclusion is that under the test conditions used the test article Ro 1525 induced chromosome aberrations in the absence of metabolic activation and Ro 1525 is considered to be clastogenic in this *in vitro* assay.

Ref.: 8

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Mammalian Erythrocyte micronucleus test

Guideline:	OECD 474
Species/strain:	Mice: Crl: NMRI BR
Group size:	5 male and 5 female in each group
Test substance:	Ro 1525
Lot no:	Ro-Rn 6567-083
Purity:	Not reported
Dose level:	50, 100 and 150 mg/kg bw
Route:	Oral gavage (only once)
Vehicle:	Deionized water
Sacrifice times:	24 h and 48 h (highest dose only)
GLP:	In compliance
Study period:	30 September – 15 November 1998

In a range-finding study, doses of 10, 50 and 200 mg test article/kg body weight were administered to two males and two females each. At the dose of 200 mg/kg bw one female was found dead after 2 hours. The rest of the animals survived 48 hours after

administration. No cytotoxicity was observed in the bone marrow evaluation. Based on these findings the test doses were chosen for the main study.

For each slide the ratio of nucleated cells to erythrocytes was determined by counting at least 200 cells per slide (at least 400 cells per animal). The ratio of polychromatic to normochromatic erythrocytes was determined by counting at least 500 erythrocytes per slide (at least 1000 erythrocytes per animal). 2000 polychromatic erythrocytes per animal were counted (1000 per slide).

Results
5/15 females and 3/15 males of the highest dose groups (150 mg/kg bw) and of the high dosed spare group died within 48 hours after administration. Spare animals replaced all deceased animals of the high dose group. In the dose group (100 mg/kg bw) 1/5 females died after 24 hours. No mortality occurred in the low dose group (50 mg/kg bw). Thus, the females were slightly more sensitive to the effects of the test article regarding mortality.

There were no indications of increases in the amount of micronucleated polychromatic erythrocytes in the dosed groups compared to the control either 24 or 48 hours treatment time and neither for males nor for females. Among the males there were no differences in the PCE/NCE ratio between the tested doses and the control. Among the females, slightly lower ratios of PCE/NCE compared to the control at all three doses were observed, indicating mild cytotoxicity.

Conclusion
Under the test conditions used the test article Ro 1525 did not induce micronuclei in the bone marrow cells of mice in this *in vivo* assay.

Ref.: 9

General comment on mutagenicity
Only 2 *in vitro* mutagenicity tests were submitted where the SCCP Notes of Guidance requires 3 *in vitro* mutagenicity tests. In the absence of positive *in vitro* studies, an additional *in vivo* test is not considered appropriate. Therefore, an *in vitro* mammalian cell gene mutation test is required.

3.3.7. Carcinogenicity

No data submitted

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

No data submitted

3.3.8.2. Teratogenicity

Pilot study

The maternal and developmental toxicity of Ro 1525 were assessed in the rat during gestation.
Ro 1525 was administered daily by oral gavage to females from Day 6 through Day 15 of gestation at a dosage of 120 mg/kg/day. Control animals received the vehicle alone (distilled water). The females were killed on gestation Day 20 and subjected to a post-mortem examination. The number of corpora lutea, weight of intact gravid Uterus, number and distribution of live foetuses, number and distribution of intra-uterine deaths, and individual foetal weight and sex were determined. All foetuses were examined externally.

The only treatment related sign observed was a reduction in body weight, body weight gain and in corrected body weight in treated females. No foetal embryo-toxicity or teratogenicity was evident at this dose level. The data suggest that the high dosage for the main study could be 120 mg/kg/day.

Main study

Guideline:	OECD 414
Species/strain:	Sprague-Dawley rats
Group size:	mated rats in four groups (n= 25)
Test substance:	RO 1525
Batch:	Ro Rn 6567-083
Purity:	> 98%
Dose:	0, 60, 80 and 120 mg/kg bw/day
Route:	oral in distilled water
Exposure:	from day 6 through day 15 of gestation
GLP:	in compliance
Study period:	9 September – 2 October 2000

Three groups of mated rats received RO 1525 by oral gavage at doses of 60, 80 and 120 mg/kg bw day in distilled water from day 6 through day 15 of gestation. A control group of 25 mated rats received the vehicle only. The day of mating was designated as day 0 of gestation. Animals were observed twice daily for morbidity/mortality. Clinical signs were checked daily. Food consumption and body weight gain were recorded. On day 20 of gestation, the animals were killed and examined macroscopically and subjected to necropsy to determine several ovary and uterine related parameters. Foetuses were weighed, sexed and examined for possible external abnormalities.

Results
A total of four females proved not to be pregnant at necropsy (1 of the control, 1 of the high dose and 2 of the mid dose group). In addition, in the high dose group one animal showed total resorption at necropsy and four animals died between gestation Day 9 and 14. The treatment related clinical signs observed in animals of the high dose group were hunched posture, decreased activity and emaciation.
In the time period of gestation day 9 and 12, food consumption showed statistically significant reductions in the high dose group. There was a statistically significant reduction of body weight seen in animals of the high dose and mid dose groups starting from gestation day 9.
There were no treatment related macroscopic findings at necropsy of the adult females. Pre-implantation litter data did not show any dosage-related trends. No specific findings were seen in foetuses.

Conclusions
The NOAEL of materno-toxicity was 60 mg/kg bw day, while the NOAEL for embryo-toxicity/teratogenicity was 120 mg/kg bw.

Ref.: 14, 15

3.3.9. Toxicokinetics

No data submitted

3.3.10. Photo-induced toxicity

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

2,2'-methylenebis-4-aminophenol
(Oxidative / permanent)

Maximum absorption through the skin	**A (µg/cm^2)**	**=**	**9.04 µg/cm²**
Skin Area surface	**SAS (cm^2)**	**=**	**700 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**6.33 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.11 mg/kg bw**
No observed adverse effect level (90-day, oral, rat)	**NOAEL**	**=**	**5 mg/kg bw**
Margin of Safety	**NOAEL / SED**	**=**	**45**

2,2'-methylenebis-4-aminophenol
(Non-oxidative / semi-permanent)

Maximum absorption through the skin	**A (µg/cm^2)**	**=**	**19.69 µg/cm²**
Skin Area surface	**SAS (cm^2)**	**=**	**700 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**13.78 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.23 mg/kg bw**
No observed adverse effect level (90-day, oral, rat)	**NOAEL**	**=**	**5 mg/kg bw**
Margin of Safety	**NOAEL / SED**	**=**	**22**

3.3.14. Discussion

Physico-chemical properties
2,2'-Methylenebis-4-aminophenol HCl is used as hair dye up to a final concentration of 2.0 % on head in the presence or absence of a developer-mix.
The UV spectrum of the test material used in the teratogenicity study is different from the UV-spectrum of the same batch of 2,2'-Methylenebis-4-aminophenol HCl provided in the analytical file. Therefore, the identity of the test substance in the submitted teratogenicity study is unclear. The melting point of 2,2'-Methylenebis-4-aminophenol HCl is not reported. Impurities in 2,2'-Methylenebis-4-aminophenol HCl have not been characterised. Quantitative information on solubility of 2,2'-Methylenebis-4-aminophenol HCl in water and other solvents are not provided. Log Pow of 2,2'-Methylenebis-4-aminophenol HCl is not provided No data is provided on the stability of Methylenebis-4-aminophenol HCl in the marketed products.

General toxicity
The maximal non-lethal dose of RO 1525 was higher than 25 and lower than 200 mg/kg bw after a single oral administration in fasted rats. In a subacute exposure (28-day study) microscopic examination showed changes in the kidney of animals from all treated groups. The data of the two 90-day studies indicated that at the 15 mg/kg bw/day dose results in tubular degeneration in Wistar rats. Therefore, the NOAEL was set at 5 mg/kg bw per day.
The NOAEL of materno-toxicity was 60 mg/kg bw day because of the reduced body weight gain, while the NOAEL for embryo-toxicity/teratogenicity was 120 mg/kg bw.

Irritation / sensitisation
The neat test substance did cause mild and transient skin irritation. It was irritant to the rabbit eye.
The test substance was not a sensitiser in a Guinea pig maximisation test. As one animal was sensitised in a Buehler test, the test substance should be regarded as a skin sensitiser.

Dermal absorption
The study was considered poor because: too few chambers were used, a 0.5 cm² application area was used instead of the recommended 0.64 cm². A dose of 40 mg/cm² was used instead of the recommended 20 mg/cm².
Under oxidative conditions, an A_{max} of 9.04 µg/cm² may be used for calculating the MOS. Under non-oxidative conditions, the Amax is 19.69 µg/cm² may be used for calculating the MOS.

Mutagenicity / genotoxicity
Ro 1525 has been investigated for the induction of gene mutations in bacteria and chromosome aberrations in Chinese hamster V79 cells. Ro 1525 did not induce gene mutations in bacteria but induced chromosome aberrations in the absence of metabolic activation. This clastogenic effect could not be confirmed in an *in vivo* assay. There were no effects of Ro 1525 based on a micronucleus test of bone marrow in mice.
The submitted *in vitro* mutagenicity tests do not fulfil the SCCP Notes of Guidance requirements for genotoxicity testing of hair dyes. An *in vitro* mammalian cell gene mutation test is required.

Carcinogenicity
No data submitted

4. CONCLUSION

Because of the low margin of safety for the use in both oxidative and non-oxidative hair dye formulations the SCCP is of the opinion that 2,2'-methylenebis-4-aminophenol HCl as a hair dye ingredient up to a final on-head concentration of 2.0% in the presence or absence of a developer-mix, poses a risk to the health of the consumer.

In addition, a gene mutation potential of 2,2'-methylenebis-4-aminophenol HCl cannot be excluded.

5. MINORITY OPINION

Not applicable

6. REFERENCES

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