OPINION OF THE SCIENTIFIC COMMITTEE ON COSMETIC PRODUCTS AND NON-FOOD PRODUCTS INTENDED FOR CONSUMERS

CONCERNING

2-CHLORO-6-METHYL-3-AMINOPHENOL HYDROCHLORIDE

COLIPA n° A 94

adopted by the SCCNFP during the 25th plenary meeting
of 20 October 2003

1. Terms of Reference

1.1. Context of the question

The adaptation to technical progress of the Annexes to Council Directive 76/768/EEC of 27 July 1976 on the approximation of the laws of the Member States relating to cosmetic products.

1.2. Request to the SCCNFP

The SCCNFP is requested to answer the following questions:

* Is 2-Chloro-6-methyl-3-aminophenol Hydrochloride safe for use in cosmetic products?

* Does the SCCNFP propose any restrictions or conditions for its use in cosmetic products?

1.3. Statement on the toxicological evaluation

The SCCNFP is the scientific advisory body to the European Commission in matters of consumer protection with respect to cosmetics and non-food products intended for consumers.

The Commission's general policy regarding research on animals supports the development of alternative methods to replace or to reduce animal testing when possible. In this context, the SCCNFP has a specific working group on alternatives to animal testing which, in co-operation with other Commission services such as ECVAM (European Centre for Validation of Alternative Methods), evaluates these methods.
The extent to which these validated methods are applicable to cosmetic products and its ingredients is a matter of the SCCNFP.

SCCNFP opinions include evaluations of experiments using laboratory animals; such tests are conducted in accordance with all legal provisions and preferably under chemical law regulations. Only in cases where no alternative method is available will such tests be evaluated and the resulting data accepted, in order to meet the fundamental requirements of the protection of consumer health.

2. Toxicological Evaluation and Characterisation

2.1. General

2.1.1. Primary name

5-Amino-6-chloro-o-cresol (INCI)

NB : The dossier and the evaluation relate to 5-amino-6-chloro-o-cresol.HCl

2.1.2. Chemical names

Chemical name	:	2-Hydroxy-3-methyl-6-amino-chloro-benzene hydrochloride
CAS name	:	2-Chloro-6-methyl-3-aminophenol hydrochloride
Synonyms	:	2-hydroxy-3-chloro-4-amino-toluene hydrochloride
		6-Chloro-5-amino-o-cresol hydrochloride
		2-Chlor-6-methyl-3-aminophenol hydrochloride

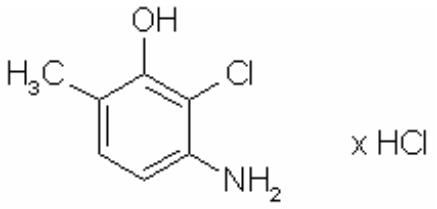
2.1.3. Trade names and abbreviations

Trade name	:	Ro 543
COLIPA No.	:	A 94

2.1.4. CAS No. / EINECS No.

CAS No.	:	80419-48-3 (hydrochloride)
EINECS	:	/

2.1.5. Structural formula



2.1.6. Empirical formula

Emp. formula	:	$C_7H_8NClO.HCl$
Mol. weight	:	194.07

2.1.7. Purity, composition, and substance codes

Test compound : 2-Chloro-6-methyl-3-aminophenol hydrochloride

Batch tested : /
Identification : by IR and UV (λ_{max} 204 nm and λ_{max} 281 nm)

Purity determined by HPLC-UV, detection at 210 nm : 94.2% (peak area)

Impurities detected by HPLC
p-Amino-o-cresol : 2.0% (peak area)
5-amino-4-chloro-2-methylphenol : 2.8% (peak area)
dichloro-derivative (no further information) : 0.8%

2.1.8. Physical properties

Appearance : Beige crystals, odourless
Melting point : 144-183°C
Boiling point : /
Density : /
Rel. vap. dens. : /
Vapour Press. : /
Log P_{ow} : /
Storage : /

2.1.9. Solubility

Water : Soluble

2.1.10. Stability

2 hours in water

General comments on analytical and physico-chemical characterisation

* Several batches of purity 80-94.2 % have been used in various studies. The HPLC purity of only one batch is reported. The batch tested for the purity has not been identified.
* Peak area count has been considered as purity/impurity criteria, absolute concentration of the dye in the test material is not reported. The HPLC detection has been performed at 210 nm, where many aromatic amines have a λ_{max}. The UV detection should have been performed by the comparison of 200-400 nm UV spectrum of the test compound with a reference material.
* Impurities are reported only in batch (purity of 94.2%). An impurity (0.8%) has been identified as dichloro-derivative. Complete identification of this molecule is not reported. The impurities (20%) in the worst case are not reported.

* No information is given about other expected impurities such as volatile materials and metals.
* Log P_{ow} of the test substance is not given.
* The method used for the determination of melting point is not reported, and no explanation is given for the large range (144-183°C) of melting point of the compound.
* Quantitative data on solubility in water is not given. Solubility in receptor fluid is not reported.
* Information provided on the stability of the test substance is insufficient.

2.2. Function and uses

2-Chloro-6-methyl-3-aminophenol hydrochloride is used as oxidative hair dye formulations up to a final concentration of 2%, after mixing with the oxidative agent.

TOXICOLOGICAL CHARACTERISATION

2.3. Toxicity

2.3.1. Acute oral toxicity

Study 1

Guideline	:	/
Species/strain	:	TNO – Wistar rat
Group size	:	10 / 20 males
Test substance	:	2-Chloro-6-methyl-3-aminophenol hydrochloride (Ro 543) dissolved in distilled water
Batch no	:	not given
Purity	:	not given
Dose	:	501, 1000, 1250, 1580, 1990 mg/kg bw by gavage
Observ. Period	:	14 days
GLP	:	/

10 male TNO – Wistar rats (mean body weight 200 g) were treated with 501, 1000, 1580 or 1990 mg/kg bw of the test substance by gavage (volume: 20 ml/kg bw), 20 male TNO – Wistar rats with 1250 mg/kg bw.

Results
Clinical symptoms observed in all dose groups were: apathy, staggering, enhanced and hindered breathing, abdominal position and yellow-orange discoloration of the urine.

The LD_{50} of the test substance administered to male rats by the oral route was 1360 (1210 – 1540) mg/kg bw.

Ref. : 1

Study 2

Guideline	:	/
Species/strain	:	Mice, CF1
Group size	:	10 males
Test substance	:	2-Chloro-6-methyl-3-aminophenol hydrochloride (Ro 543) dissolved in distilled water
Batch no	:	not given
Purity	:	not given
Dose	:	501, 631, 794, 1000, 1250, 1580, 1990 mg/kg bw by gavage
Observ. Period	:	14 days
GLP	:	/

10 male CF1 mice (mean body weight 27 g) were treated with 501, 1000, 1580 or 1990 mg/kg bw of the test substance by gavage (volume: 20 ml/kg bw).

Results
Clinical symptoms observed in all dose groups were: apathy, enhanced breathing, abdominal position, cramps and uttering of tone. The LD_{50} of the test substance administered to male rats by the oral route was 1200 (1030 – 1400) mg/kg bw.

Ref. : 2

2.3.2. Acute dermal toxicity

No data

2.3.3. Acute inhalation toxicity

No data

2.3.4. Repeated dose oral toxicity

No data

2.3.5. Repeated dose dermal toxicity

2.3.6. Repeated dose inhalation toxicity

No data

2.3.7. Sub-chronic oral toxicity

Guideline	:	/
Species/strain	:	MuRA Han 67 Wistar rats, SPF
Group size	:	10 males + 10 females
Test substance	:	1-Hydroxy-2-methyl-5-amino-6-chloro-benzene (Ro 543) in 1 % Traganth (Tragacantha DAB 7 albiss. pulv. subt.)
Batch number	:	1748/64

Purity	:	not given
Dose levels	:	0, 50 mg/kg bw by gavage
Exposure period	:	66/67 days, once daily, 5 days per week
GLP	:	/

40 rats (20 males, 137-181 g bw and 20 females, 121-168 g bw) were used. The test substance was administered by gavage, once daily 5 days per week for 66/67 days at dosage levels of 0 and 50 mg/kg bw, application volume 10 ml/kg bw. The control group received the vehicle (1 % Traganth) only. All animals were observed daily for clinical signs and mortality, body weights were recorded individually in weekly intervals. At 6 / 7 weeks and at the end of the study, blood and urine samples were taken from all animals for haematological (15 parameters) and clinical chemistry (14 parameters) investigations as well as for urinalysis (15 parameters). Ophthalmoscopic examination was performed before the end of the study on all animals. All animals were sacrificed at the end of the study, organ weights were recorded, macroscopy and histopathology were performed.

Results
One animal died during the study (female of the control group during narcosis). No changes in body weight gain were observed, no abnormal findings were noted at ophthalmoscopy. Only minor changes were found in some haematological and clinical biochemistry parameters and are of no relevance related to the test substance. In treated males, the weight of testes was slightly decreased compared to the control group. Ovary weights were not measured.

All animals suffered from a retroviral infection (Sialodacryoadenitis) and showed histo-pathological alterations related to this disease.

The NOAEL in this study is 50 mg/kg bw.

Comment
According to OECD guideline 408 only healthy animals should be used for subchronic oral toxicity testing. In addition, several dose levels and a satellite group should be included.

Ref. : 18

2.3.8. Sub-chronic dermal toxicity

No data

2.3.9. Sub-chronic inhalation toxicity

No data

2.3.10. Chronic toxicity

No data

2.4. Irritation and corrosivity

2.4.1. Irritation (skin)

Guideline : /
Species/strain : albino rabbits, New Zealand
Group size : 6 males
Test substance : Ro543
Batch number : /
Purity : /
Dose : 0.5ml of a 10% formulation *
GLP : /

24 hours prior to treatment, the dorsal fur was shaved to expose an area of about 10 cm^2. An aliquot of 0.5 ml of a 10% formulation of Ro543 was applied to the intact skin under a 3 x 3 cm pad. The patches were removed after 2 hours and observations made at the time and at 24 and 48 hours after removal.

(the formulation was described as consisting of 3 g test substance, 10 ml distilled water, 5 ml concentrated ammonia made up to 30 ml with 96% ethanol)*

Results
No cutaneous reactions were observed during the study. Ro 543 was considered to be non-irritant to rabbit skin under the conditions of the study. The test is considered to be inadequate for evaluation.

Ref. : 3

Repeated application - Mice

Guideline : /
Species/strain : hairless mice. Hr/hr strain
Group size : 6 males
Test substance : Ro543
Batch number : /
Purity :
Dose : 'drops' of a 10% formulation *
GLP : /

One drop of a 10% formulation of Ro543 was applied to the intact skin on a small area of each animal's back twice daily for 5 consecutive days.

(the formulation was described as consisting of 3 g test substance, 10ml distilled water, 5 ml concentrated ammonia made up to 30 ml with 96% ethanol)*

Results
No cutaneous reactions were observed during the study. A 10% dilution of Ro 543 was considered to be *non-irritant* to mice skin under the conditions of the study with repeated application.

Ref. : 5

Repeated application - Rabbits

Guideline	:	/
Species/strain	:	Rabbits. New Zealand
Group size	:	6 male
Test substance	:	Ro543
Batch number	:	/
Purity	:	/
Dose	:	'drops' of a 10% formulation *
GLP	:	/

One drop of a 10% formulation of Ro543 was applied to the intact skin on a small shaved area of each animal's back. The application was repeated every 30 seconds for 30 minutes to the same area of skin.
Observations were made after the applications and at 24 and 48 hours.

(the formulation was described as consisting of 3 g test substance, 10ml distilled water, 5 ml concentrated ammonia made up to 30 ml with 96% ethanol)*

Results
No cutaneous reactions were observed during the study. A 10% dilution of Ro 543 was considered to be *non-irritant* to rabbit skin under the conditions of the study with repeated application.

Ref. : 6

2.4.2. Irritation (mucous membranes)

Guideline	:	/
Species/strain	:	albino rabbits, New Zealand
Group size	:	6 males
Test substance	:	Ro543
Batch number	:	/
Purity	:	/
Dose	:	0.1ml of 5% aqueous solution
GLP	:	not in compliance

A volume of 0.1ml of 5% aqueous test substance was instilled into the conjunctival sack of the right eyes of the test animals. The substance remained in permanent contact with the eyes. The left eyes served as controls.
2, 6, 24, and 48 hours after instillation of the test material, the treated eyes of the rabbits were observed for signs of ocular irritation.

Results
There were no effects observed on the iris or cornea. Conjunctival injection was observed in all animals but remained to 24 hours in only one animal. Conjunctival oedema was present in 3 animals but persisted to 6 hours in one animal.

Ref. : 4

2.5. Sensitisation

Magnusson and Kligman Guinea pig maximisation test

Guideline	:	/		
Species/strain	:	guinea pigs, Pirbright White		
Group size	:	40 females (20 test and 20 control)		
Test substance	:	Ro543		
Batch number	:	/		
Purity	:	/		
Dose	:	Intradermal induction	:	0.1 ml of 5% aqueous solution, Freund's Complete Adjuvant and equal parts of these two into either side of dorsal region.
		Topical induction	:	5% dilution of test material in white soft paraffin under occlusion for 48 hours. Controls received vehicle only *
		Challenge	:	14 days after epidermal applications with 25% dilution in white soft paraffin (24 hours, occlusion).
GLP	:	not in compliance		

Animals were examined 24 and 48 hours after removal of the patches for signs of erythema and oedema.

Results
5 of the animals showed erythema 24 hours after the challenge application. These reactions had gone by 48 hours. These reactions were interpreted as irritant.

Comment
The study is considered inadequate.

*A non-irritant dilution of the test substance was used for induction. There was no pre-treatment with SLS.

Ref. : 7

2.6. Teratogenicity

Guideline	:	OECD 414 (1981)
Species/strain	:	Wistar/HAN rat, SPF-quality
Group size	:	25 females mated per dose group
Test substance	:	Ro 543 in distilled water
Batch number	:	2665 / 196
Purity	:	90 %
Dose levels	:	0, 30, 90, 270 mg/kg bw daily by gavage
Treatment period	:	Day 6 - 15 of gestation
GLP	:	in compliance

The test substance was administered orally, once daily by gavage, from day 6 to 15 of gestation (volume: 10 ml/kg bw) to groups of 25 pregnant rats (184 – 234 g) at dose levels of 0, 30, 90 and 270 mg/kg bw. The control group received the vehicle (distilled water) only. All mated

females were sacrificed on day 21 of gestation. The animals were observed at least twice daily for mortality and clinical signs. Individual body weights were recorded daily from day 0 to 21 post coitum. Food consumption was measured for the day-intervals 0-6, 6-11, 11-16, and 16-21. Immediately following sacrifice, macroscopic examination of the maternal organs was carried out. The uterus was removed and weighed, the number of corpora lutea, early and late resorptions, total implantations and viable foetuses and their distribution and site in the uterus were recorded. All foetuses were individually weighed and the sex of the foetuses was determined. One half of the foetuses was examined for skeletal defects and variations of the ossification process by Alizarin Red staining and one half was evaluated for visceral alterations.

Results
No treatment-related macroscopic changes were detected in females at terminal necropsy. The corrected body weight gain in dams was reduced in group 3 (90 mg/kg bw) and group 4 (270 mg/kg bw) compared to the control group and slight effects on food consumption were reported for these groups.
No substance-related changes of reproduction data (number of implantations, resorptions and foetuses, foetal weight and external abnormalities) were noted. No substance-related changes in the incidence of visceral and skeletal abnormalities were found.

The NOAEL of maternal and embryo/foetotoxicity was 30 mg/kg bw in this study.

Ref. : 19

2.7. Toxicokinetics (including Percutaneous Absorption)

2.7.1. Percutaneous Absorption *in vivo*

Guideline	:	not documented (report prepared in 1988)
Tissue	:	Rat clipped dorsal skin (female rats Wistar strain)
Method	:	*in vivo* measurement of the absorption from the urinary and faecal excretion, and from the skin residue and carcass analysis after sacrifice of the animal
Test substance	:	2-chloro-6-methyl-3-aminophenol HCl, in a commercial type formulation (pH 9.5) at the concentration of 1.14 % applied on the skin Study performed without a developer and with a developer (H_2O_2)
Batch no	:	/
Dose	:	200 mg of formulation over 10 cm²
Replicate	:	12 rats for the formula without a developer and 6 rats for the formula with a developer
Analyt. method	:	liquid scintillation, ring ^{14}C-2-chloro-6-methyl-3-aminophenol HCl
GLP	:	not in compliance

The skin penetration of 2-chloro-6-methyl-3-aminophenol HCl was evaluated in vivo in rats. The test substance was prepared in a “commercial type” formulation. Before the application the hair dye was mixed with water “study excluding a developer” or with a developer solution containing 3 % hydrogen peroxide “study including a developer”. The final concentration of 2-chloro-6-methyl-3-aminophenol HCl was 1.14 %.
200 mg of the final formulation were applied to 10 cm² of the dorsal skin. For the study excluding a developer the contact with the substance was maintained during 2 days. For the study including a developer the exposure was limited to 30 minutes.

Urine and faeces were collected during 2 days, the animals were then sacrificed, the skin of the application site and the carcass were analysed separately. ^{14}C-1,10-bis-(2,5-diaminophenyl)-1,4,7,10-tetraoxadecane (x4 HCl) and related compounds were globally assayed by liquid scintillation.

Results
For the formula "without a developer" and an exposure of 2 days, the quantity of test substance penetrating through the skin, evaluated from the urinary and faecal excretions and from the amount recovered in the whole carcass was 93.2 % of the applied dose. Corrections were made because of the very high mass balance (115 %) and the "corrected absorption" was estimated to be 81 % of the applied dose. The elimination was via urine (87.7 % - uncorrected value) and most of the radioactivity was eliminated within the first 24 hours. The residual amount in the carcass was marginal (0.477 %).
For the formula with the developer, the recovery rate of the experiment was at the bottom acceptable level (85 %). So the total absorption corrected for the low mass balance after an exposure limited to 30 minutes was estimated to be 0.116 % of the applied dose. The residual amount still present in the skin was 0.501 % of the applied dose, the one in the carcass was 0.033 % of the applied dose.

This test was performed *in vivo* in the rat, it is technically corresponding to the current OECD guidelines. The interest of such a measurement in rat and the experimental differences between the two experiments performed are questionable. The study does not conform to the SCCNFP recommendations. Because of the tremendous differences obtained when the product is applied with or without a developer in the formula, and because of the discrepancy between the 30 minutes exposure for the formulation with the developer and the 2 day exposure for the formulation without the developer, a skin penetration study should be performed according to the SCCNFP Notes of Guidance.

Ref. : 13, 14

2.7.2. Distribution and elimination *in vivo*

Study 1

Guideline	:	/
Species/strain	:	Wistar rats SPF-quality
Group size	:	5 males
Test substance	:	ring-labelled *[^{14}C]* 2-chloro-6-methyl-3-aminophenol hydrochloride, specific activity 382 MBq/mMol, radiochemical purity > 98 %, in distilled water
Dose levels	:	10 mg/kg bw subcutaneous, single dose
Observation time:		4 days
GLP	:	not in compliance

Five male rats (254-270 g) were used for this assay. The radioactive compound was administered subcutaneous as a single dose of approximately 10 mg/kg bw applied as 1 g solution in the neck. Excretion patterns were studied for 96 hours after application. Radioactivity was determined in urine and faeces, for 2 animals the expired air was additionally investigated. Urine and CO_2 measurements were carried out for intervals of 0 – 8 h, 8 – 24 h, 24 – 48 h, 48 – 72 h and 72 – 96 h; faeces was sampled as daily fraction. Rats were sacrificed after the end of observation.

Results
Most of the radioactivity was measured in the urine (78.2 – 95.2 %), while 1.4 – 7.8 % was detected in the faeces. Minor amounts of radioactivity (< 0.1 %) were found in the skin at application area and as residuals in the carcass (0.4 – 1.6 %). No radioactivity could be detected in expired air. The mean value for recovery was 99.0 %.

Renal excretion occurred mainly within 8 h after application (mean value: 76.2 %). 24 h after application the mean value for cumulative excretion was 91.5 % (88.1 % in the urine, 3.4 % in the faeces).

Ref.: 15

Study 2

Guideline	:	/
Species/strain	:	Wistar rats SPF
Group size	:	5 males
Test substance	:	ring-labelled *[^{14}C]* 2-chloro-6-methyl-3-aminophenol hydrochloride, specific activity 382 MBq/mMol, radiochemical purity > 98 %, in distilled water
Dose levels	:	50 mg/kg bw oral by gavage, single dose
Observation time:		4 days
GLP	:	not in compliance

Five male rats (321 - 336 g) were used for this assay. The radioactive compound was administered orally per gavage as a single dose of approximately 50 mg/kg bw. Excretion patterns were studied for 96 hours after application. Radioactivity was determined in urine and faces, for 2 animals the expired air was additionally investigated. Urine and CO_2 measurements were carried out for intervals of 0 – 8 h, 8 – 24 h, 24 – 48 h, 48 – 72 h and 72 – 96 h; faeces was sampled as daily fraction. Rats were sacrificed after the end of observation.

Results
Most of the radioactivity was found in the urine (83.5 – 101.3 %), while 2.1 - 11.3 % was detected in the faeces. Minor amounts of radioactivity (< 0.1 %) were found as residuals in the gastro-intestine tissue (0.021 – 0.057 %) and in the carcass (0.54 - 0.66 %). No radioactivity could be detected in expired air. The mean value for recovery was 105.8 %.

Renal excretion occurred mainly within 8 h after application (mean value: 75.9 %). 24 h after application the mean value for cumulative excretion was 99.4 % (95.1 % in the urine, 4.3 % in the faeces).

Ref.: 16

Study 3

Guideline	:	/
Species/strain	:	Wistar rats, BOR:WISW, SPF-quality
Group size	:	5 males
Test substance	:	ring-labelled *[^{14}C]* 2-chloro-6-methyl-3-aminophenol hydrochloride, specific activity 382 MBq/mMol, radiochemical purity > 98 %, in distilled water

Dose levels : 50 mg/kg bw oral by gavage, single dose
Observation time : 4 days
GLP : not in compliance

Five male rats (315-333 g) were used for this assay. The test substance was administered orally per gavage as a single dose of approximately 50 mg/kg bw. Organ distribution was investigated by autoradiography 1 h, 6h, 24h, 48h and 96h after application. Rats were sacrificed after the end of observation.

Results
1 h after application mainly the stomach content but also the skin, kidney, bladder and the intestine parts were labelled. Only minor contents were measured in the liver. After 6 h the labelling was increased in the intestine but still predominant in the stomach. After 24 and 48 h a noteworthy labelling was detected only in the intestine and 96 h after application only residual radioactivity was measured in bones.

Ref.: 17

2.8. Mutagenicity/Genotoxicity

2.8.1. Mutagenicity/Genotoxicity *in vitro*

Bacterial Reverse Mutation Test

Guideline : /
Species/strain : *Salmonella typhimurium,* TA98, TA100, TA1535, TA1537, TA 1538
Replicates : Triplicate plates, 2 independent tests
Test substance : Ro 543 dissolved in DMSO/bidistilled water
Batch no : /
Purity : /
Concentrations : Experiment # 1 and # 2
Salmonella typhimurium and *E. coli*
With or without metabolic activation
Test #1 : 4, 20, 100, 500, 2500 µg/plate
GLP : not in compliance

A 94 (2-hydroxy-3-chloro-4-aminotoluene) has been investigated for gene mutation in *S. typhimurium* using the direct plate incorporation method both with or without S9 mix. Purchased S9 mix was obtained from rats injected i.p. with Aroclor^{TM} 1254 or with Phenobarbital.

Results

Toxicity : no information.

Revertant number
Test # 1 & Test # 2
* In the absence of activation, a dose related relevant decrease in revertant numbers was observed. The biological relevance of these observations should have been considered with regard to cytotoxicity if any. Cytotoxicity could have prevented the expression of revertant.

* In the presence of "Aroclor" activation : increase in revertant numbers was observed in the TA 100 tester strain in both experiments. However, the amplitude of the results are different between the first and second experiment. Because the results were pooled, only a slight increase was observed for TA 100.
* Increases in mutant frequencies have been observed for the top doses for the tester strains TA 1538 and TA 98.
* Positive controls showed the expected response in the absence of activation system. No positive controls have been used in the presence of activation.
* In the presence of "phenobarbital" activation : increases in mutant frequency was observed in TA 100 and TA 1537 and TA 1538 for the lower doses followed by a decrease in MF. While not always statistically significant, the shape of the induced results indicates a mutagenic potential of Ro 543.

Conclusions
Based on the reversion rate, and under the conditions of the assays performed (in the absence of activation; one in the presence of Aroclor induced S9 mix and one in the presence of phenobarbital induced S9 mix), it is concluded that the test agent Ro 543 in the presence of S9 mix, shows evidence of mutagenic activity in some tester strains. In the absence of activation systems, a decrease in the revertant numbers was observed and may be the result of cytotoxicity, the latter parameter having not be taken into account.
The test is considered unacceptable for evaluation due to the following reasons : purity and batch not given, enzyme activity of both activation systems (Aroclor and Phenobarbital S9 fraction were not checked for their activity); no concurrent adequate positive control was included in the presence of activation, the assays were not conducted in compliance with GLP or OECD guidelines.

Ref. : 8

In Vitro Mammalian Cell Gene Mutation Test

OECD guideline	:	OECD 476 (1984)
Species/strain	:	V79 cell line / HPRT Locus
Replicates	:	2 independent tests with and without metabolic activation
Test substance	:	Ro 543 dissolved in DMSO
Batch no	:	2665/122
Purity	:	80 – 90 % pure substance
		10 – 20 % other substances
Stability	:	pure : unknown
	:	in solvent : unknown
Concentrations	:	Experiment # 1 and # 2
		Without metabolic activation : 35, 100, 200 and 350 µg/ml
		With metabolic activation : 25, 100, 200, 1000 and 3000 µg/ ml
		Experiment # 2
Treatment time	:	4 hours
GLP	:	In compliance

A 94 (2-hydroxy-3-chloro-4-aminotoluene) has been investigated for gene mutation in the HPRT locus of V79 Chinese hamster cell lines, both with or without S9 mix. S9 mix was obtained from male Wistar rats injected i.p. with Aroclor^{TM} 1254. Negative and positive controls were in accordance with the OECD guideline.

Results
Solubility : not described

Osmolarity
Osmolarity measurement of post treatment medium was not performed.

Plating efficiency
Test # 1 & 2 with or without S9 mix
Relative Plating efficiency was reduced at acceptable levels for the top doses (Exp # 1 - without S9 : 25 %, with S9 : 18 % and Exp # 2 - without S9 : 24 %, with S9 : 18.9 %).

Mutant frequencies
In the absence or presence of activation, no dose related significant increase in mutant colony numbers was observed.

Conclusions
No biologically relevant significant increase in mutant colony numbers was observed over the concurrent solvent controls after treatment with 2-hydroxy-3-chloro-4-aminotoluene in either test in the presence or absence of activation. Therefore, the test substance does not demonstrate mutagenic potential on the HGPRT locus in V79 cells.

Ref. : 9

***In vitro* mammalian chromosomal aberration test**

Guideline	:	OECD 473 (1983)		
Species/strain	:	Chinese Hamster V79 Cells		
Replicates	:	Duplicate cultures, 2 independent experiments		
Test substance	:	Ro 543 dissolved in DMSO		
Batch no	:	2665/196		
Purity	:	± 85 %		
Stability	:	pure : stable for years		
	:	in solvent : several hours in water, not stable in alkaline solution		
Treatment time	:	4 hours		
Concentrations	:	Test without S9 :	1100 µg/ml	7 hours
			10, 500, 800 µg/ml	18 hours
			1100 µg/ml	28 hours
		Test with S9 :	1100 µg/ml	7 hours
			10, 500, 800 µg/ml	18 hours
			1100 µg/ml	28 hours
GLP	:	In compliance		

A 94 (2-hydroxy-3-chloro-4-aminotoluene) has been investigated for chromosomal aberrations in V79 Chinese hamster cell lines, both with or without S9 mix. S9 mix was obtained from male Wistar rats injected i.p. with Aroclor^{TM} 1254. Negative and positive controls were in accordance with the OECD guideline.

Exposure
The test material has been added during 4 hours to exponentially growing cultures in the presence or absence of S9 mix : a) after 48 h cultures : 7 and 28 hours preparation interval;

b) after 55 h cultures : 18 hours preparation interval.

Results
Toxicity : At the top dose levels, a clear reduction of the plating efficiency and of the Mitotic Index was noted.

Structural chromosome aberrations
A statistically, biologically relevant and dose dependent significant increase in the numbers of aberrant cells was observed as compared to the corresponding solvent control for all doses and at any preparation intervals but for the 7 hours interval with S9 mix.
Qualitatively speaking, the aberrant cells displayed fragments and many exchange figures (up to 73 %).

Polyploidy : not taken into account

Conclusions
A biologically relevant significant increase in the numbers of aberrant cells was observed over the concurrent solvent controls after treatment with 2-hydroxy-3-chloro-4-aminotoluene in either test in the presence or absence of activation. Therefore, the test substance displays clastogenic properties in V79 cells.

Ref. : 10

2.8.2 Mutagenicity/Genotoxicity *in vivo*

Mammalian Erythrocyte Micronucleus Test

Guideline	:	OECD 474 (1981)
Species	:	OF1 mice
Group sizes	:	5 males and 5 females
Test substance	:	Ro 543 dissolved in sterile water
Batch no	:	2665/122
Purity	:	Not described
Dose levels	:	The test was administered by 1 single oral dose of 1200 mg/kg bw for 24, 48 and 72 h sacrifice time
GLP	:	In compliance

A 94 (2-hydroxy-3-chloro-4-aminotoluene) has been investigated for induction of micronuclei in the bone marrow cells of male or female mice. A preliminary range finding study in which observable clinical toxic effects were seen at doses of 1200 mg/bw determined the dose level. The substance was administered by a single intragastric gavage and the groups of animals sacrificed 24, 48 or 72 hours after administration. Negative and positive controls were in accordance with the OECD guideline.

Number of cells scored : a total of at least 1000 erythrocytes were examined from each animal and the incidence of micronucleated erythrocytes determined. The ratio of polychromatic erythrocytes to normochromatic erythrocytes was calculated after the microscopic observations of 400 cells only.
Results
NCE/PCE

* The mean number of the ratio was slightly increased after treatment as compared to controls. The biological significance of this observation is difficult to establish; only 400 cells were scored (normally 1000 – 2000) and the results may reflect the inter-individual variability.
* Micronucleated PCE 24, 48 or 72 hours sampling time : no statistically significant or biologically relevant increase in the incidence of micronucleated polychromatic erythrocytes over the concurrent vehicle control values were observed for any dose levels.

Conclusions
Under the conditions of the test it can be concluded that A 94 (2-hydroxy-3-chloro-4-aminotoluene), at a dose at which some signs of clinical toxicity were recorded, does not induce statistically significant increase in the frequency of micronucleated PCE.
Toxico-kinetic studies do indicate that the compound reaches the bone marrow cells at a biological relevant dose. The compound is non-genotoxic in the *in vivo* micronucleus test.

Ref. : 12

Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *in vitro*

Guideline	:	OECD 482
Species/strain	:	Wistar rats CF HB hepatocytes.
Test substance	:	Ro 543 dissolved in culture medium
Batch no	:	2665/196
Purity	:	± 85 %
Stability	:	pure : stable for years
	:	in solvent : several hours in water, not stable in alkaline solution
Dose levels	:	Exp # 1 6.7, 66.67, 100.0, 333.33 and 666.7 µg/ml
		Exp # 2 6.7, 66.67, 100.0, 666.7 and 1000 µg/ml
		Exp # 3 600, 1000, 1300, 1600 and 2000 µg/ml.
Exposure time	:	3 hours in the presence of ^{3}H-thymidine.
GLP	:	in compliance

A 94 (2-hydroxy-3-chloro-4-aminotoluene) was tested for induction of UDS in freshly isolated hepatocytes in three independent experiments. Hepatocytes were incubated with the test material and ^{3}H-thymidine for three hours, then nuclear DNA was isolated and the incorporation of ^{3}H-thymidine was determined by liquid scintillation counting. The test article was dissolved in L-15 culture medium. 2-Acetylaminofluorene served as positive control.

Results
UDS assay Exp #1 No reduction in the incorporation of radioactivity occurred at any concentration but the top dose (negative ctrl 129.2 ± 12.2 dpm/µg DNA; top dose of 666.67 µg/ml : 2984.00 ± 16.7 dpm/µg DNA).
Exp #3 A slight increase in the incorporation of radioactivity, occurred for the middle and high dose, this was followed by a decrease of radioactivity.
From the results the authors concluded that A 94 (2-hydroxy-3-chloro-4-aminotoluene) did not induce a significant increase in DNA repair in freshly isolated rat hepatocytes.

Conclusions
Only one exposure period has been evaluated (3 h) and no indication about the potential effects due to a long term exposure (14 – 16 h) is presented. Although not specifically disallowed by the OECD guideline, the use of liquid scintillation counting is considered inferior to

autoradiographic scoring of UDS, because of potential interference from cells undergoing replicative DNA synthesis.
The study is inadequate.

2.9. Carcinogenicity

No data

2.10. Special investigations

No data

2.11. Safety evaluation

Not applicable

2.12. Conclusions

The information on impurities in the test material is incomplete. Insufficient information is provided on physico-chemical characterisation, solubility and stability of the test material

In a subchronic oral toxicity study in rats the NOAEL was 50 mg/kg day/bw. But, the study did not follow OECD guidelines. The teratogenicity study in rats revealed a NOAEL of both maternal and embryo/foetal toxicity of 30 mg/kg bw.

The test substance was considered to be non-irritating to the skin and the eye. The sensitisation study was considered inadequate.

Percutaneous absorption studies have been carried out at concentrations below the applied in use concentration of 2 %.

2-hydroxy-3-chloro-4-aminotoluene hydrochloride has been tested in prokaryotic and mammalian cells for gene mutation, and in mammalian cells for chromosomal aberration *in vitro*. An *in vitro* UDS assay has been performed and one *in vivo* bone marrow micronucleus test has been performed.
The *in vitro* test for gene mutation in prokaryotes with the test agent Ro 543 has been found positive in the presence of metabolic activation system. However, the assay is considered unsuitable for evaluation.
The *in vitro* test for gene mutation in mammalian cells showed that the test agent is non mutagenic under both activation conditions. However, the batch is different from the one used in the prokaryotes study and the purity low (85 %), in addition no information about the nature of impurities is given.
The *in vitro* test for clastogenicity in Chinese hamster V79 cells is clearly positive, both in the presence or in the absence of activation system. Such properties are confirmed by the large increase in the number of cells carrying exchanges figures.
The *in vivo* micronucleus test in mice gave negative results.
The *in vitro* induction of UDS in freshly isolated hepatocytes has been analysed. However, the study is inadequate due to the technological methodology selected and the absence of long exposure time.

The data provided on mutagenicity/genotoxicity is insufficient for a final evaluation.

2.13. References

1. Potokar, M. (1992) 2-Chlor-6-methyl-3-aminophenol hydrochlorid - Akute orale Toxizität an Ratten. Henkel KGaA, Düsseldorf, Report No. RT 920197
2. Potokar, M. (1992) 2-Chlor-6-methyl-3-aminophenol hydrochlorid - Akute orale Toxizität an Mäusen. Henkel KGaA, Düsseldorf, Report No. RT 920199
3. Potokar, M. (1992) 2-Chlor-6-methyl-3-aminophenol hydrochlorid - Prüfung auf primäre Hautirritation. Henkel KGaA, Düsseldorf, Report No. RT 920202
4. Potokar, M. (1992) 2-Chlor-6-methyl-3-aminophenol hydrochlorid - Prüfung auf primare Schleimhautirritation. Henkel KGaA, Düsseldorf, Report No. RT 920204
5. Potokar, M. (1992) 2-Chlor-6-methyl-3-aminophenol hydrochlorid - Prüfung auf primäre Hautirritation bei wiederholter cutaner Applikation. Henkel KGaA, Düsseldorf, Report No. RT 920203
6. Potokar, M. (1992) 2-Chlor-6-methyl-3-aminophenol hydrochlorid - Prüfung auf primäre Hautirritation bei wiederholter Benetzung der Haut. Henkel KGaA, Düsseldorf, Report No. RT 920201
7. Potokar, M. (1992) 2-Chlor-6-methyl-3-aminophenol hydrochlorid - Prüfung auf sensibilisierende Eigenschaften an Meerschweinchen. Henkel KGaA, Düsseldorf, Report No. RT 920212
8. Banduhn, N. (1993) 2-Hydroxy-3-chlor-4-aminotoluol (Ro 543) - Salmonella/Mammalian Microsome Mutagenicity Test (Ames Test). Henkel KGaA, Düsseldorf, Report No. RT 930094
9. Heidemann, A.. (1988) Detection of gene mutations in mammalian cells in vitro (HGPRT test) with Ro 543. CCR, Cytotest Cell Research GmbH & Co. KG, Rossdorf, Project 116605 sponsored by Henkel KGaA, Düsseldorf, Report No. R 9400936
10. Heidernann, A.(1989) Chromosome Aberration Assay in Chinese Hamster V79 Cells in vitro with Ro 543. CCR, Cytotest Cell Research GmbH & Co. KG, Rossdorf, Project 132715 sponsored by Henkel KGaA, Dtisseldorf, Report No. EX 0194
11. Weill, N. (1989) Ro 543 - Micronucleus Test in the Mouse. Hazleton France, L'Arbresle/F, Report No 908303 sponsored by Henkel KGaA, Düsseldorf, Report No. EX 0271
12. Timm, A.(1988) Unscheduled DNA synthesis in hepatocytes of male rats in vitro with Ro 543. CCR Cytotest Cell Research GmbH & Co. KG, CCR Project 132726 sponsored by Henkel KGaA, Düsseldorf, Report No. R 9400937
13. Bartnik, F. & Zimmermann V. (1988) 2-Chlor-6-methyl-3-aminophenol (Ro 543) - Ermittlung der cutanen Resorption der Reinsubstanz aus einer Basiscreme. Henkel KGaA, Düsseldorf, Report No. TBD 880899
14. Bartnik, F.&Zimmerman, V.(1988) 2-Chlor-6-methyl-3-aminophenol (Ro 543) - Ermittlung der cutanen Resorption aus zwei Haarfarbstofformulierungen - Versuche mit Ratten. Henkel KGaA, Düsseldorf, Report No. TBD 890040
15. Bartnik, F.& Zimmermann, V.(1987) 2-Chlor-6-methyl-3-aminophenol - Ermittlung des Ausscheideverhaltens nach einmaliger subcutaner Applikation. Henkel KGaA, Düsseldorf, Report No. TBD 870336
16. Bartnik, F. & Zimmermann V. (1987) 2-Chlor-6-methyl-3-aminophenol (Ro 543) - Intestinale Resorption nach einmaliger oraler Applikation. Henkel KGaA, Düsseldorf, Report No. TBD 870387
17. Bartnik, F. Pittemann, W. & Zimmermann, V. (1987) 2-Chlor-6-methyl-3-aminophenol - Ermittlung der Organverteilung mittels Ganztierautoradiographie nach oraler Applikation Henkel KGaA, Abschlussbericht, Archiv-Nr. TBD 870603

18. Potokar, M., Pittermann, W. & Bartnik, F.(1986) 1-Hydroxy-2-methyl-5-amino-6-chlor-benzo1 - 90-Tage-Test nach wiederholter oraler Verabreichung an Ratten. Henkel KGaA, Düsseldorf, Report TBD 860044
19. Becker, H., Frei, D., Mladenovic, P. & Terrier, Ch. (1988) Embryotoxicity study (including teratogenicity) with Ro 543 in the rat. RCC Research & Consulting Company, CH-Itingen, Project No 204491, sponsored by Henkel KGaA, Düsseldorf, Report No. FX 0246

3. Opinion of the SCCNFP

The SCCNFP is of the opinion that the information submitted is inadequate to assess the safe use of the substance.

Before any further consideration, the following information is required :

* Complete chemical characterisation (purity and impurities) of all batches of the test material; Log P_{ow} , solubility in relevant solvents including the receptor fluid used for percutaneous absorption study, stability of the test material in test solutions and hair dye formulations.

* percutaneous absorption study in accordance with the SCCNFP Notes of Guidance.

* data on the genotoxicity/mutagenicity following the relevant SCCNFP-opinions and in accordance with the Notes of Guidance.

4. Other considerations

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5. Minority opinions

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