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

CONCERNING

HC BLUE N° 7

COLIPA n° A130

adopted by the SCCNFP during the 23rd plenary meeting
of 18 March 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 HC Blue n° 7 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

HC Blue n° 7 (INCI name)

2.1.2. Synonyms

6-Methoxy-N^2-methylpyridine-2,3-diamine dihydrochloride (EINECS, IUPAC)
Oxidinblau
3-Amino-2-methylamino-6-methoxypyridine
6-Methoxy-N^2-methyl-2,3-pyridinediamine
Pyridinblau

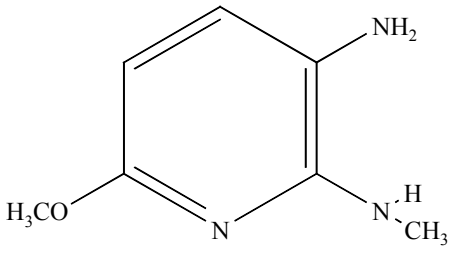
2.1.3. Trade names and abbreviations

Ro 730
COLIPA n° A130

2.1.4. CAS n° / EINECS n°

CAS n° : 90817-34-8 (base)
83732-72-3 (dihydrochloride)
EINECS n° : 280-622-9 (dihydrochloride)

2.1.5. Structural formula



+ 2 HCl

2.1.6. Empirical formula

Emp. Formula : $C_7H_{11}N_3O$ x 2HCl
Mol weight : 226.11 (dihydrochloride)

2.1.7. Purity, composition and substance codes

sA : 3-Amino-2-methylamino-6-methoxypyridine, dihydrochloride (> 99.0%)

2.1.8. Physical properties

Appearance	:	grey to brown powder, characteristic odour
Melting point	:	201-204 °C with decomposition
Boiling point	:	no information
Density	:	no information
Rel. vap. dens.	:	no information
Vapour Press.	:	no information
Log P_{ow}	:	no information

2.1.9. Solubility

Soluble in water, slightly soluble in alcohol

General comments on analytical and physico-chemical characterisation

* Log Pow and density not given
* No quantitative data given on solubility

2.2. Function and uses

3-Amino-2-methylamino-6-methoxypyridine dihydrochloride is used in oxidative hair dye formulations at a maximum concentration of 2%. The substance is mixed with hydrogen peroxide before application, the use concentration is 1%.

TOXICOLOGICAL CHARACTERISATION

2.3. Toxicity

2.3.1. Acute oral toxicity

Overview

Species	Sex	LD-50 (mg/kg bw)	Reference
mouse	female	813	1
rat	male	700	1
rat	female	650	1
mouse	female	1355	2

3-Amino-2-methylamino-6-methoxypyridine dihydrochloride, dissolved in water, was administered once via stomach tube to 10 CF1 female mice and Wistar rats (6/sex). Mice were dosed with 625, 700, 775, 850 or 925 mg/kg bw and rats received doses of 600, 700, 800 or 900 mg/kg bw.

During an observation period of 14 days, the mortalities and clinical-toxicological findings were recorded daily and the body weights were noted weekly. A post mortem examination was carried out in all animals. The test substance caused reduced activity, piloerection, diarrhoea and exitus.

Results

Species	**Sex**	**No.**	**Dose (mg/kg)**	**Mortality**
mouse	female	10	625	1/10
		10	700	2/10
		10	775	1/10
		10	850	6/10
		10	925	10/10
rat	female	6	600	3/6
		6	700	5/6
		6	800	4/6
		6	900	6/6
rat	male	6	600	0/6
		6	700	5/6
		6	800	4/6
			900	6/6

Ref.: 1

3-Amino-2-methylamino-6-methoxypyridine dihydrochloride, dissolved in 2% carboxymethylcellulose and 0.5% Cremophor, was administered once via stomach tube to 10 CF1 male mice. Mice were dosed with 794, 1000, 1250, 1415, 1580 or 1990 mg/kg bw. During an observation period of 14 days, the mortalities and clinical-toxicological findings were recorded daily and the body weights were noted weekly. A post mortem examination was carried out in all animals. The test substance caused increased breathing and tonic and clonic convulsions.

Results

Species	**Sex**	**No.**	**Dose (mg/kg)**	**Mortality**
mice	male	10	794	0/10
		10	1000	1/10
		10	1250	3/10
		10	1415	6/10
		10	1580	7/10
		10	1990	9/10

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

Guideline	:	/
Species/strain	:	Wistar rat
Group size	:	15 male +15 female
Test substance	:	3-AMMP
Dose levels	:	75, 150 and 300 mg/kg bw, 5 days/week by gavage
Exposure period	:	3 weeks
GLP	:	not in compliance

The test substance was administered, by gavage, once daily to 4 groups (15/sex) (bw males 204-272 g; bw females 160-202 g) for 3 weeks. The test substance was administered at dosage levels of 75, 150 or 300 mg/kg bw. The control group received the vehicle (aqua dest.) only. All animals were sacrificed at the end of the study.

All animals were observed daily for mortality and clinical signs. Body weights, food and water consumption were recorded individually in weekly intervals. Blood samples were taken from 10 rats/sex of all groups for haematological (8 parameters) and clinical chemistry (6 parameters) investigations, on days 0 and 21. Urine samples (8 parameters) were taken from 5 rats/sex of all test groups, on days 0 and 21. Macroscopy and histopathology (5 organs/tissues) was performed, on all animals.

Results
No animal died during the study. A discoloration of the urine was observed in all test groups. In the highest dose group a significant reduction of the food consumption was observed, only in the first week of the study. In females a significant decrease in the body weight even in the low dose group (75 mg/kg bw) was observed whereas in the males the body weights decreased only in the highest dose group (day 7 and 14). In the highest dose group, crust formation in the stomach was observed. In the mid and highdose group LDH, GOT (only mid dose males) and AP were significantly decreased (not dose-related). Changes in several blood parameters (RBC, HB, HCT, MCH, MCHC) were observed even in the low dose group (75 mg/kg bw), but no dose-response relationship was seen. Slight pale discoloration of some thyroid glands without any deposit of pigment was observed in all test groups (not dose-related). Alterations in the hormone status of the thyroid glands (T3 decreased and T4 increased) were observed. With males a significant T3 decrease was found even in the low dose group. Histomorphologically, in the highest dose group an increased activity of the follicular epithelium of thyroid gland was observed. Atrophy of the testes was observed in 4/15 rats of the highest dose group.

No dose level without adverse effects was found in this study, the LOAEL was 75 mg/kg bw.
Ref.: 9

2.3.5. Repeated dose dermal toxicity

No data

2.3.6. Repeated dose inhalation toxicity

No data

2.3.7. Sub-chronic oral toxicity

Guideline : /
Species/strain : Wistar rat
Group size : 10 male, 10 female
Recovery group : 5 male, 5 female
Recovery period: 4 weeks
Test substance : Ro 730
Dose levels : 0 and 50 mg/kg bw, 5 days/week by gavage
Exposure period : 90 days
GLP : not in compliance

The test substance was administered, by gavage, once daily 5 days/week, to Wistar rats (10/sex) (age: 5 weeks; bw males 72-105 g; bw females 75-112 g) for 90 days at dosage levels of 0 or 50 mg/kg bw. The control group received the vehicle (aqua bidest) only. For recovery observations, satellite groups of 5 male and 5 female rats were attached to the two dose groups and observed for 4 weeks without treatment. All animals were sacrificed at the end of the study.

All animals were observed daily for mortality and clinical signs. Body weights and food consumption were recorded individually in weekly intervals. Blood samples were withdrawn from all animals of each test group for haematological and clinical chemistry investigations (thyroid function not studied), during week 7 and 13 (17). Urine samples were taken from 5 males and 5 females of each test group, during week 6, 12 and 16. Organ weights were measured and macroscopy and histopathology was performed, on all animals.
No animal died during the study. No signs of toxicity were observed. The repeated dosing led to mild, reversible irritations of the cutaneous region of the forestomach. However, this finding was not regarded as an indication of systemic toxicity. But, in all substance groups for both sexes an increase in the number of thrombocytes was observed which was calculated to be significant for females (7 weeks) and males (13 weeks), respectively. No dose level without effects was found.

Remark
This subchronic toxicity study was not conducted in accordance with OECD 408, because only 1 dose and 1 control group were used (at least 3 dose levels and a control should be used). This study is considered inadequate. Moreover, in the subacute toxicity study clear-cut effects (dose-related) on the thyroid function (target organ) were observed as well as some indication for haematotoxicity. In the 90 days study the latter effect was confirmed whereas no parameters of thyroid function were investigated.

Ref.: 12

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 & corrosivity

2.4.1. Irritation (skin)

Study 1

Guideline	:	/
Species/strain	:	New Zealand albino rabbit
Group size	:	3 females
Test substance	:	3-AMMP
Dose	:	0.5 g, undiluted
GLP	:	not in compliance

0.5 g of the undiluted test substance (patch moistened with reverse osmosis water) was applied semi-occlusively to the right, clipped back of 3 female New Zealand rabbits for 4 hours. The substance residues were washed off. Observations for signs of dermal irritation were recorded 1, 24, 48 and 72 hours and 7 days after patch removal.

Very slight erythema was observed in 1 animal at 6 hr post application. In one animal, also 3 brown spots with crust formation was observed. The irritation was reversible within 7 days after exposure. The Draize score was 0.3 (not irritating).

Ref.: 4

Study 2

Guideline	:	/
Species/strain	:	Hairless mice, hr/hr strain
Group size	:	10 females
Test substance	:	3-AMMP
Dose	:	1-2 drops of a 10% (w/v) suspension in 2% carboxymethylcellulose and 0.5% Cremophor in water
GLP	:	not in compliance

One to two drops of a 10% (w/v) suspension of the test substance in 2% carboxymethyl-cellulose and 0.5% Cremophor in water, were applied twice daily for five days to the same area of the clipped back of 10 female hr/hr mice.

Observations for signs of dermal irritation were recorded 1, 2, 3, 4 and 5 days after application. No signs of irritation were observed. The score was 0.0 (not irritating).

Ref.: 5

Study 3

Guideline	:	/
Species/strain	:	human
Group size	:	10 adult men and women
Test substance	:	3-AMMP
Dose	:	One drop of a 10% (w/v) solution in 2% carboxymethylcellulose and 0.5% Cremophor in water, repeatedly in 30 min on 5 cm^2
GLP	:	not in compliance

One drop of a 10% aqueous solution (w/v) of the test substance was applied repeatedly at intervals of 30 seconds over a period of half an hour to an area of 5 cm^2 of the forearm skin (volar side) of 10 human volunteers.

Observations for signs of dermal irritation were recorded till 30 minutes after application. After 60 applications no signs of irritation were observed in 8/10 volunteers. Two volunteers felt a slight burning accompanied by a slight erythema. The reference substance, carboxymethylcellulose, induced 3 reactions in 10 volunteers participating in the test.

Ref.: 6

2.4.2. Irritation (mucous membranes)

Guideline	:	/
Species/strain	:	New Zealand albino rabbit
Group size	:	6 males
Test substance	:	3-AMMP
Dose	:	0.1 ml of a 5% solution of the test substance in 2% carboxymethylcellulose and 0.5% Cremophor in water
GLP	:	not in compliance

0.1 ml of a 5% solution of the test substance in 2% carboxymethylcellulose and 0.5% Cremophor in water, was instilled into the conjunctival sac of the right eyes of 6 male albino New Zealand rabbits. The untreated left eyes served as controls. The eyes were examined 1, 6, 24, 48 and 72 hours after application. No signs of irritation were observed. The Draize score was 0.0 (not irritating).

Ref.: 3

2.5. Sensitisation

Magnusson and Kligman study

Guideline	:	/
Substance	:	3-AMMP
Species/strain	:	Pirbright guinea pig
Group size	:	20 female + 20 female in control group

Test substance	:	3-AMMP	
Concentrations	:	intradermal induction :	0.1 ml FCA
			0.1 ml 5% test substance in propylene glycol
			0.1 ml 5% test substance in FCA (1:1)
		challenge :	1 % in propylene glycol for 24 h, occluded
GLP	:	not in compliance	

Two groups of 20 female Pirbright white guinea pigs (1 control and 1 test group) were used in this skin sensitisation study. The induction phase consisted of 3 series of 2 intradermal injections in the clipped dorsal shoulder region of each animal. The intradermal injections were divided as follows: 2 injections of 0.1 ml of a 5% solution of the test substance in FCA (1:1), 2 injections of 0.1 ml of a 5% solution of the test substance in propylene glycol and 2 injections of 0.1 ml FCA. The control group received the vehicle (propylene glycol, FCA).
Day 1-6: examination on local tolerance.
Day 7, an epicutaneous induction of the test substance (5%) in vaseline. The occlusive patch application lasted for 48 hours on the surface corresponding to the intradermal injections. The control group received vaseline.
Day 12-20: rest period. On day 21, the challenge phase started; the left shoulder was treated with the test substance (1%) in propylene glycol in a 24 hours occlusive patch test, while the right shoulder was treated with the vehicle. Skin reactions are evaluated 24 and 48 hours after the end of the challenge exposure.

Results
No challenge skin reactions were observed.

Ref.: 7

2.6. Teratogenicity

Guideline	:	/
Species/strain	:	Wistar rats (BOR:WISW-SPF TNO)
Group size	:	24 females mated per dose group
Test substance	:	3-AMMP in water
Dose levels	:	0, 5, 15 or 30 mg/kg bw by gavage
Treatment period	:	Day 5 - 15 of gestation
GLP	:	in compliance

The test substance was administered, once daily by gavage, from day 5 to 15 of gestation to 4 groups of 24 pregnant rats at dosage levels of 0, 5, 15 or 30 mg/kg bw. The control group received the vehicle (aqua dest.) only. All mated females were sacrificed at day 20 of gestation.

The animals were observed daily for clinical signs. Individual body weights were recorded at days 0, 5, 10, 15 and 20. Food consumption was measured for the day-intervals 0-5, 5-15, 15-20 and 0-20. Immediately following sacrifice, the uterus was removed, weighed and the number of (non)viable foetuses, early and late resorptions and the number of total implantations and corpora lutea was recorded. A macroscopic examination of the organs was carried out. All foetuses were individually weighed and the sex of the foetuses was determined. Two third of the foetuses was examined for skeletal defects and variations of the ossification process by Alizarin Red staining and one third was evaluated for visceral alterations.

Results
The fur of all animals in control and test groups appeared smooth and brightly. A slightly reduced food consumption (not significant) was observed in the highest dose group. No substance-related irreversible structural changes were found. The dose level without maternal and without embryo/foetotoxicity was 30 mg/kg bw.

Ref.: 15

2.7. Toxicokinetics (incl. Percutaneous Absorption)

2.7.2. Percutaneous absorption, distribution and elimination *in vivo*

Study 1

Guideline : /
Species/strain : 5 male + 5 female Wistar rats (SPF-Cpb)
Test substance : [^{14}C]3-AMMP, purity: > 96% in a basic cream*
Dose levels : 0.034% (as free base) after addition of hydrogen peroxide
Exposure time : 24 h
GLP : in compliance

The test substance was applied to the clipped dorsal skin of Wistar rats (SPF-Cpb) (5/sex) for 48 hours and then washed off. The test substance was integrated in a basic cream (without developer). The concentration of [^{14}C]3-AMMP was 0.1 % (0.068% as 3-AMMP base) before 1:1 dilution, after addition of hydrogen peroxide the concentration of [^{14}C]3-AMMP (base) was 0.034%. The amount of test substance applied per animal was 200 mg. The content of radioactivity was determined in rinsing water, treated skin areas, urine, faeces, expired air, organs and carcass.

Results
88.3% (males) and 73.9% (females) of the applied radioactivity was removed from the skin by rinsing 48 hours after the beginning of the cutaneous application. The treated area of the skin still contained a small fraction of the administered ^{14}C-radioactivity: 0.834% (males) and 1.08% (females). Small ^{14}C-concentrations were found in the organs (stomach and the caecum) after 24 hours (0.620% in males and 1.34% in females). A value of 0.267% for the male and 0.264% for the female rats were found in the expired air (measured with 2 rats/sex). The absorbed amount of ^{14}C-labelled test substance was excreted mainly via urine (9.24% for males and 17.2% for females) and to a lesser extent via faeces (2.39% for males and 5.64% for females). Most of the radioactivity was eliminated within the first 24 hours after application. The mean percutaneous absorption rate of the test substance, was 12.9% for male and 25.9% for female rats.

* Composition of the cream:

Substance	**g**
[^{14}C]3-AMMP	0.01
Fatty alcohol	1.05
Lorol, technical	0.24
sodium laurylether sulphate (26.5%)	3.10
ammonium sulphate	0.10
sodium sulphite	0.10

ammonia (25%)	0.25
water	10.00

Remark
Inadequate study. The test concentration is far below the expected use concentration of 1.0 %.
Ref.: 10

Study 2

Guideline : /
Species/strain : 5 male + 5 female Wistar rats (SPF-Cpb)
Test substance : [^{14}C]3-AMMP, purity: > 96% in a hair dye formulation*
Dose levels : 0.034% (as free base) after addition of hydrogen peroxide
Exposure time : 30 min
GLP : in compliance

[^{14}C]3-AMMP was applied, in a formulation similar to those normally used for hair dyeing, to the clipped dorsal skin of Wistar rats (SPF-Cpb) (5/sex) for 30 minutes, and then washed off with 100 ml shampoo-solution. After addition of hydrogen peroxide the concentration of [^{14}C]3-AMMP (base) was 0.034%. The amount of test substance applied per animal was 200 mg. The content of radioactivity was determined in rinsing water, treated skin areas, urine, faeces, organs and carcass.

Results
65.8% (males) and 72.3% (females) of the applied [^{14}C] radioactivity was removed from the skin by rinsing 48 hours after the beginning of the cutaneous application. The treated area of the skin still contained a fraction of the administered [^{14}C] radioactivity: 30.2% (males) and 21.5% (females). Small ^{14}C-concentrations were found in the organs (stomach and the caecum) after 24 hours (0.094% in males and 0.129% in females). The absorbed amount of [^{14}C] radioactivity was excreted via urine (0.42% for males and 0.61 % for females) and via faeces (0.63% for males and 1.01% for females). Most of the radioactivity was eliminated within the first 24 hours after application. 0.079 µg/cm^2 skin of 3-AMMP equivalents (1.2% of the applied radioactivity, male rats) and 0.124 µg/cm^2 of 3-AMMP equivalents (1.87% of the applied radioactivity, female rats) were percutaneously absorbed in a 48 h period.

* Composition of the formulation:

Substance	**%**
[^{14}C]3-AMMP	0.10
fatty alcohol	12.90
sodium laurylether sulphate (26.5%)	31.00
ammonium sulphate	1.00
sodium sulphite	1.00
resorcinol	0.23
p-amino-o-cresol	0.077
chlororesorcinol	0.25
p-tolulenediamine sulphate	1.08

ammonia (25%)	3.52
water	ad 100.00

Remark
Inadequate study. The test concentration is far below the expected use concentration of 1.0 %.

Ref.: 11

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
Substance : A 130
Batch no : not given
Purity : not given
GLP : not in compliance

Liver S9 fraction from liver rats pre-treated with Aroclor 1254 was used as the exogenous metabolic activation system.

Results
Toxicity was noted at the top dose tested (2500 µg/plate). Negative results were found in the presence or the absence of metabolic activation system in the experiment performed..

Conclusions
Based on the reversion rate, it is concluded that the test agent A 130 does not show evidence of mutagenic activity in the presence or in the absence of metabolic activation system in several *Salmonella typhimurium* tester strains.
However, the experiment was not replicated, the purity batch is not given, the guidelines and GLP attestation are not described. It should be noted that AT tester strains have not been used as requested by OECD guidelines 471 (TA102).
The study is considered inadequate.

Ref. : 16

***In Vitro* Mammalian Cell Gene Mutation Test**

Guideline : /
Species/strain : L5178Y cell line / $TK^{+/-}$ Locus
Replicates : 2 independent tests with and without metabolic activation
Substance : Imexine OBA
Batch no : Pil.10
Purity : not given
Treatment time: : 2 hours
GLP : not in compliance

Liver S9 fraction from male Wistar rats pre-treated with Aroclor 1254 was used as the exogenous metabolic activation system.

Results
First experiment
At 5 and 20 µg/ml in the absence of activation system, the compound shows statistically significant positive effects but without dose-effect relationship. In addition the increases were only observed in one of the replicates. Taken together, those increase may be considered as devoid of biological significance. In the presence of activation, no increase in mutant frequency has been observed.

Conclusions
From the results generated in only one experiment, it may be concluded that A 130 give negative results in this test. However, purity is not given, no independent repeat experiment was performed.

Ref. : 17

In Vitro Mammalian Cell Transformation Test

Guideline	:	/
Species/strain	:	Syrian Hamster embryos cells
Replicates	:	yes
Substance	:	Oxidinblau
Batch no	:	/
purity	:	99 %
Exposure time	:	6 h and 48h without S9 mix, 6 h with activation system.
Doses	:	With S9 mix: 1, 10, 50 or 100 µg/ml Without S9 mix: 0.1, 0.5, 1 or 2 µg/ml
GLP	:	in compliance

Liver S9 fraction from male Wistar liver rats pre-treated with Aroclor 1254 was used as the exogenous metabolic activation system.

Results
Under both conditions – with or without S9 mix - the test agent A 130 did not induce a statistically and/or biologically significant increase in the number of transformed colonies.
Conclusions
COLIPA A 130 has been investigated for its ability to induce cell transformation in Syrian Hamster Embryos cells. The results are negative.

Ref. : 21

DNA Damage and Repair-Unscheduled DNA Synthesis-Mammalian Cells _In Vitro_

Guideline	:	OECD 482
Species/strain	:	HeLa cells
Replicates	:	No
Test substance	:	Purity 101.6 % given by NaOH titer
Exposure tme	:	2.5 h

Doses : +/- S9 mix : 0.0064, 0.032, 0.16, 0.8, 4, 20, 100 or 500 µg/ml
GLP : in compliance

Results
COLIPA A 130 has been investigated for induction of unscheduled DNA synthesis in HeLa cells. Liver S9 fraction from liver rats pre-treated with Aroclor 1254 was used as the exogenous metabolic activation system.
Under both conditions in the presence or absence of activation system, a weak but statistically significant increase and dose related unscheduled DNA synthesis was noted (expressed as dpm/µg DNA). However, the methodology adopted (scintillation counting) is less sensitive, according to the scientific literature.

Conclusion
The UDS-test *in vitro* had positive results.

Ref. : 22

2.8.2. Mutagenicity/Genotoxicity *in vivo*

In Vivo Sister Chromatid Exchanges

Guideline : /
Species/strain : Sprague-Dawley rats
Replicates : no
Substance : A 130
Batch no : /
Administration : single intraperitoneal injection
Doses : 10, 30, 100, 150 or 200 mg/kg bw
GLP : not in compliance

Results
First experiment
At the top dose of 200 mg/kg bw, 3/8 animals died immediately.
A slight dose related increase in SCEs frequencies was observed from 100 mg/kg bw onwards.

Conclusion
The study provided gives positive results. However, the individual values are given with the standard error of mean while the group mean values are noted with the standard deviation. In addition, only 25 cells per animal have been scored. Moreover, the use of 5-BrdU allows to differentiate between cells having passed through 1, 2 or more DNA synthesis phases. This allows to check the proliferation rate index and gives indications on cytotoxicity or mitotic delay. Such information are not given in this study.

The study is considered inadequate. The purity of the compound and the batch are not given.

Ref. : 18

In Vivo Sister Chromatid Exchange Assay

Guideline : /
Species/strain : Sprague-Dawley rats

Replicates	:	no
Substance	:	A 130
Batch no	:	/
Administration	:	single oral gavage
Doses	:	6, 20,50, 100, 150, 200 or 600 mg/kg bw
GLP	:	not in compliance

Results

First experiment

At the dose of 50 mg/kg bw., a slight statistically significant increase was observed. No dose-effect relationship was evident.

Conclusion

The study provided gives positive results at only one dose (50 mg/kg bw).
However, the individual values are given with the standard error of mean while the group mean values are noted with the standard deviation. In addition, only 25 cells per animal have been scored. Moreover, the use of 5-BrdU allows to differentiate between cells having passed through 1, 2 or more DNA synthesis phases. This allows to check the proliferation rate index and gives indications on cytotoxicity or mitotic delay. Such information are not given in this study. The purity of the compound and the batch are not given. The study result is considered equivocal, and the study inadequate.

Ref. : 19

***In Vivo* Sister Chromatid Exchanges**

Guideline	:	/
Species/strain	:	Chinese Hamsters
Replicates	:	none
Substance	:	A 130
Batch no	:	/
Administration	:	single intraperitoneal injection
Doses	:	25, 50, 75, 100, 150 or 200 mg/kg bw.
GLP	:	not in compliance

Results

First experiment

At the dose of 100 mg/kg bw. and onwards mortality was observed.
No statistically dose related increase in SCEs frequencies was observed . However, the sample size of each treated groups is different (n= 5, 5, 6, 4, 2, 2; from low to high doses). This induces bias.

Conclusion

The study provided gives negative results. However, the individual values are given with the standard error of mean while the group mean values are noted with the standard deviation. In addition, only 25 cells per animal have been scored. Moreover, the use of 5-BrdU allows to differentiate between cells having passed through 1, 2 or more DNA synthesis phases. This allows to check the proliferation rate index and gives indications on cytotoxicity or mitotic delay. Such information are not given in this study.

The purity of the compound and the batch are not given, in addition sample size are not similar. This study is not acceptable.

Ref. : 20

Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *In Vivo*

Guideline	:	OECD 486 (1981)
Species/strain	:	Wistar rats
Group size	:	4 males
Test substance	:	A 130 in deionized water
Batch no	:	/
Dose levels	:	60 and 600 mg/kg bw, by gavage
Exposure time	:	16 hours: all dose groups; 2h: high dose group
GLP	:	in compliance

COLIPA A 130 has been investigated for induction of unscheduled DNA synthesis in Wistar rats hepatocytes at 2 doses 60 and 600 mg/kg. Positive controls are in accordance with OECD guideline and UDS analysed by autoradiography. 4 males were used per dose/time sampling.

Results
No difference in viability of treated rats hepatocytes has been observed as compared to controls. No evidence of UDS induced by the test agent was observed.

Conclusions
This study is adequate and the results negative.

Ref. : 24

***In Vivo* Mammalian Erythrocyte Micronucleus Test**

Guideline	:	OECD 474
Species/strain	:	Mouse, Albino CF1/W 68
Group size	:	5 male + 5 female
Test substance	:	Ro 730 in 1% carboxymethylcellullose
Batch no	:	Pil.10 (purity not stated in study report)
Dose levels	:	0, 100, 500 and 1000 mg/kg bw
Sacrifice times	:	6 hours post dosing
Administration	:	Two repeated oral gavage
GLP	:	in compliance

COLIPA A 130 has been investigated for induction of micronuclei in the bone marrow cells of Albino CF1/W 68. The substance was administered twice by intragastric gavage at 100, 500 and 1000 mg/kg bw, the bone marrow was harvested 6 h after last dosing. Negative and positive controls were in accordance with the OECD guideline.

Results
No statistically significant or biologically relevant increase in the incidence of micronucleated polychromatic cells over the concurrent vehicle control values was observed. However, it should be noted that a large difference in micronucleated cells was observed between male and female

animals. Moreover, one sampling time of 6 h may not be sufficient for observing induction of micronuclei.
No mice treated with COLIPA A 130 exhibited changes of the PCE/NCE ratio and it not clear that the test substance has reached bone marrow.

Conclusion
Under the conditions of the test, it can be concluded that there was no evidence of induced chromosomal or other damage leading to micronucleus formation in polychromatic erythrocytes of treated mice. But, the study is inadequate

Ref. : 23

***In Vivo* Mammalian Bone Marrow Chromosome Aberration Test**

Guideline	:	OECD 475 (1997)
Species	:	Wistar Rats (SPF)
Sex	:	6 males and 6 females / group
Test substance	:	Batch n. 96003970 (SAT010561:HPLC)
Purity	:	99.7%
Positive control	:	CPA 15 mg/kg bw (24 h)
Treatment	:	24 and 48 h
Dose	:	50 mg/kg bw ip
GLP	:	in compliance

Results
No statistical significant increase of chromosome aberrations in the animals treated for 24 and 48 h was observed. The positive control (CPA) induced 14.6% chromosome aberration. A reduction of Mitotic Index was observed, thus indicating the exposure of bone marrow cells to the chemical. The chemical is considered non clastogenic *in vivo* in the rats.

General conclusion on mutagenicity

A 130 has been tested in procaryotic cells for gene mutation in several tester strains in an inadequate study that gave negative results. The *in vitro* test for mammalian gene mutation assay is accepted as being negative. The three inadequate *in vivo* tests for sister chromatid exchanges on rats (2 experiments) and on hamsters (1 experiment), have produced equivocal results. The cell transformation assay in Syrian Hamster Embryos cells gave negative results. UDS in human HeLa cells *in vitro* gave positive results. UDS *in vivo/in vitro* on rats hepatocytes gave negative results. The *in vivo* micronucleus test in mice gave negative results; no firm evidence that the bone marrow was reached by the test agent was however noted. The study is inadequate because only one time was evaluated. An *in vivo* study on the induction of chromosome aberration on rats (bone marrow) indicated negative results. When considering all results, the compound A 130 is considered as devoid of genotoxic, mutagenic and clastogenic potentials *in vivo*, based on the two studies submitted.

2.9. Carcinogenicity

No data

2.10. Special investigations

Excretion after subcutaneous administration

Five male Wistar rats were used as test group. 10 mg [^{14}C] 3-AMMP per kg bw (purity: 96%) was applied as a single subcutaneous dose. The excretion in urine, expired air and in the carcass was studied for 96 hours at 8-hour intervals. For the faeces : 24 hour intervals.

Results
54.2% of the administered radioactivity was excreted in the urine within 96 hours after application. Within the first 24 hours 49.2% had already been excreted. 0.188% of the administered dose was found in the expired air. In the faeces 26.8% of the applied dose was eliminated within 96 hours, 20.2% within the first 24 hours. 2.5% of the administered dose was found in the carcass.

Ref.: 12

Excretion after oral administration in rats

Five male Wistar rats were used as test group. 11.2 mg [^{14}C] 3-AMMP per kg bw (purity : > 96%) was administered, by gavage, as a single dose. The excretion in urine, faeces and expired air (8 hour intervals and faeces in 24 hour intervals) and the amount of radioactivity remaining in the carcass and in the gastrointestinal tract was determined for 96 hours.

Results
49.4% of the administered radioactivity was excreted in the urine within 96 hours after application. Within the first 8 hours 32.4% had already been excreted. 0.017% of the administered dose was found in the expired air. In the faeces 42.9% of the applied dose was eliminated within 96 hours. 0.79% and 0.08% of the applied dose was found in the carcass and gastro-intestinal tract at the end of the study.

Ref.: 13

Organ distribution following oral administrations in rats

Five male Wistar rats were used as test group. 10 mg [^{14}C] 3-AMMP per kg bw (purity : > 96%) was administered, by gavage, as a single dose. 0.5, 1, 2, 6 and 24 hours after administration, the kinetic of the organ distribution was recorded by whole body autoradiography.

Results
30 and 60 minutes after application the stomach and the small intestine were labelled intensively; compared with these two organs the kidneys and the liver were less labelled.
At the end of the study the stomach and the caecum were still labelled relatively intensively, the liver was only slightly discernible in the autoradiographs. In all other organs no radioactivity could be detected.

Ref.: 14

2.11. Safety evaluation

NOT APPLICABLE

CALCULATION OF THE MARGIN OF SAFETY

HC Blue n° 7
(Oxidative hair dye)

Based on a usage volume of 100 ml, containing at maximum … %

Maximum absorption through the skin	A ($\mu g/cm^2$)	=	$\mu g/cm^2$
Typical body weight of human		=	60 kg
Skin Area surface	SAS (cm^2)	=	cm^2
Dermal absorption per treatment	SAS x A x 0.001	=	mg
Systemic exposure dose (SED)	SAS x A x 0.001/60	=	mg/kg
No observed adverse effect level (mg/kg) (species, study)	NOAEL	=	mg/kg

Margin of Safety	NOAEL / SED	=

2.12. Conclusions

HC Blue n° 7 is moderately toxic, on the basis of its acute oral toxicity.

A 5% and 100% solution of HC Blue n° 7 was not irritating to the eye and skin of rabbits. A 10% suspension of HC Blue n° 7 was not irritating to the skin of mice, after repeated application. A 10% solution of HC Blue n° 7 was repeatedly tested on the skin of human volunteers. No sign of irritation was observed in 8/10 volunteers, however 2/20 volunteers felt a slight burning accompanied with slight erythema. The Magnusson & Kligman test showed no evidence of sensitisation.

In a 21-day toxicity study with rats, effects were found at all doses. The 90-day toxicity study with rats is inadequate. In a teratogenicity study with rats, no substance-related irreversible structural changes were observed.

The percutaneous absorption studies are inadequate.

Based on the adequate provided mutagenicity studies, HC Blue n° 7 is not considered to be genotoxic and clastogenic *in vivo*.

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3. Opinion of the SCCNFP

The SCCNFP is of the opinion that the information submitted is insufficient to allow an adequate risk assessment to be carried out. Accordingly, the SCCNFP considers that it is not possible to assess the safe use of the substance.

Before any further consideration, the following information is required :

* The NOAEL has to be defined based on a subacute or subchronic toxicity study according to current guidelines including investigations on haematotoxicity and of thyroid function.

* A study on percutaneous absorption according to the Notes of Guidance (SCCNFP/0321/00);

* data on the genotoxicity/mutagenicity following the SCCNFP-opinion “Proposal for a Strategy for Testing Hair Dye Cosmetic Ingredients for their Potential of Genotoxicity / Mutagenicity”, doc. n° SCCNFP/0566/02 of 4 June 2002, and in accordance with the Notes of Guidance, regularly updated by the SCCNFP (doc. n° SCCNFP/0321/00).

4. Other considerations

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5. Minority opinions

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