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

CONCERNING

BASIC RED 51

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 Basic Red 51 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

Basic Red 51 (INCI)

2.1.2. Chemical names

Chemical name	:	2-[((4-Dimethylamino)phenyl)azo]-1,3-dimethyl-1H-imidazolium chloride
CAS name	:	1H-Imidazolium, 2-[((4-dimethylamino)phenyl)azo]-1,3-dimethyl-, chloride
Synonyms	:	/

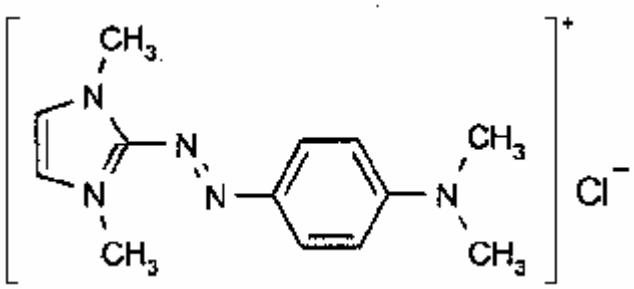
2.1.3. Trade names and abbreviations

Trade name	:	Vibracolor Ruby Rot; MIP 2985
COLIPA n°	:	/

2.1.4. CAS No. / EINECS No.

CAS no	:	77061-58-6
EINECS	:	278-601-4

2.1.5. Structural formula



2.1.6. Empirical formula

Emp. Formula	:	$C_{13}H_{18}ClN_5$ (as chloride)	$C_{13}H_{18}ClN_5O_4$ (as perchlorate)*
Mol weight	:	279.6 g/mol (chloride)	343.6 g/mol (perchlorate)*

* used for quantification of the dye in the test materials

2.1.7. Purity, composition, and substance codes

Purity

Titre as determined by HPLC	:	97.2 - 98.6 % respectively (an overall deviation of 5% is assumed due to lumps)
Water content	:	0.3-1.2 %
Heavy metals	:	/
Potential impurities	:	≤ 0.4 % coloured product of unknown identity
Reagents and intermediate reaction products	:	/
Solvent residues	:	0.1 % isopropanol

2.1.8. Physical properties

Appearance	:	blue to dark violet powder containing lumps
Melting point	:	>350°C (decomposition)
Boiling point	:	/
Density	:	/
Rel. vap. dens.	:	/
Vapour Press.	:	/
Log P_{ow}	:	–1.97 (OECD method No. 197/1981)

2.1.9. Solubility

In water : 40 g/l at 30°C

2.1.10 Stability

No data

General comments on analytical and physico-chemical characterisation

* A discrepancy of the salt content in the samples has been noted. During the manufacturing process, the dyestuff is salted out with NaCl. Vacuum drying and recrystallisation will produce high purity dye with less salt content.
* Impurity of a colour product, content up to 0.4%, has not been characterised.
* No experimental data is provided on stability of the test material.

2.2. Function and uses

Intended for use in direct hair dye formulations at concentrations up to 0.2% and in oxidative hair dyes at a final concentration of 0.1%, after mixing with the oxidative agent.

TOXICOLOGICAL CHARACTERISATION

2.3. Toxicity

2.3.1. Acute oral toxicity

Guideline	:	/
Species/strain	:	Rat, Crl : CD (SD)IGS BR
Group Size	:	2 rats per sex at the 3 low doses, 5 rats per sex at the highest dose
Test material	:	MIP 2985
Batch no	:	029753 A8AA
Purity	:	97.2%
Dose	:	500, 1000, 1500, 2000 mg/kg bw
Observ. period	:	14 days
GLP	:	in compliance

In a dose-limit test, the test substance was dissolved in cell culture grade water and administered by gavage as single doses of MIP 2985 at 2000 mg/kg bw (5 rats per sex). The treatment-related mortality occurred at this dose. Thus additional dose levels (500, 1000, 1500) were tested on 2 rats per sex per dose.
Most animals, except one female and 2 males at the lowest dose (500 mg/kg bw), died within the first 24 h of the experiment. The female died on Day 1. Clinical observations observed prior to death included hypoactivity, recumbency, irregular respiration, ataxia, and squinted eye.

The 2 males from the lowest dose (500 mg/kg bw) survived until the scheduled sacrifice and gained weight during the course of the study. Clinical observations in these animals included hypoactivity, irregular respiration, ataxia, squinted eyes, discoloured urine, faecal stains, and/or few faeces. Most of these findings were resolved by Day 3.

At necropsy, no visible lesions were noted in the two males that survived in the 500 mg/kg dose group. In the animals that died, macroscopic findings involved the stomach, caecum, ileum, duodenum, jejunum, and/or colon. These were discoloured and/or distended filled with purple, red, or pink fluid.

Lower Dose Study

Guideline	:	/
Species/strain	:	Rat, Crl : CD (SD)IGS BR
Group Size	:	5 male rats at the high dose only, 5 female rats at the low dose only
Test material	:	MIP 2985
Batch no	:	029753 A8AA
Purity	:	97.2%
Dose	:	250, 500 mg/kg bw
Observ. period	:	14 days
GLP	:	in compliance

In this small range-finding study, dosages were reduced. 2 males died on Day 1. All other animals survived until the end of the experiment.

3 males, including the two that died on Day 1, were hypoactive and had roughened haircoat within 4 hours. Macroscopic findings of the 2 males that died, showed discoloured and/or distended stomach, caecum, ileum, duodenum, jejunum and bladder filled with purple, red, or pink fluid. 1 female was hypoactive and had liquid and/or mucoid faeces within 4h post-dosing. All surviving animals gained weight and showed no post-mortem anomalies at the end of the study. The acute oral LD_{5O} was considered to be between 250 - 500 mg/kg bw in females and between 500 - 1000 mg/kg bw in males.

Ref. : 1

2.3.2. Acute dermal toxicity

Guideline	:	OECD Guideline 402
Species/strain	:	Rat, Crl : CD (SD)IGS BR
Group Size	:	10 males and females
Test material	:	MIP 2985
Batch no	:	029753 A8AA
Purity	:	97.2%
Dose	:	2000 mg/kg bw
Observ. period	:	14 days
GLP	:	in compliance

The test material was moistened with distilled water and applied at a dose of 2000 mg/kg bw. The hair was clipped the day prior to the experiment. It was applied to the clipped area as a thin uniform layer from scapula to iliac crest and half way down the flank on each side of the animal's back. The area was occluded for 24 h. The initial dermal irritation was scored and recorded 30 minutes after bandage removal on Day 1. The untreated skin of each animal served as the control. Additional dermal irritation readings were performed for each animal on Days 3, 7, 10, and 14. All animals were examined for clinical signs of ill health or mortality immediately post-dose and approximately 1, 2.5 and 4 hours post-dose, and daily thereafter. Body weights were recorded pre-dose on the day of dosing (Day 0), and on Days 7 and 14, and prior to sacrifice on Day 15 (fasted). A curtailed gross examination of the cervical, thoracic and abdominal viscera was performed.

Signs of clinical toxicity included chromodacryorrhea and/or red nasal discharge. Findings were first noted 4 hours post-dose and were resolved by Day 2. Signs of dermal irritation included desquamation (slight scaling) in all males on Day 3 and in one male and one female on Day 7. There were no signs of dermal irritation at any observation interval in any of the remaining animals. All animals gained weight during the course of the study. No visible lesions were noted in any of the animals at necropsy.

MIP 2985 was mildly irritating to the skin when applied dermally at a dose of 2000 mg/kg bw. Under the conditions of this study, there were also minor systemic effects. The acute dermal LD_{5O} is greater than 2000 mg/kg bodyweight.

Ref. : 2

2.3.3. Acute inhalation toxicity

No data

2.3.4. Repeated dose oral toxicity

Guideline	:	OECD Guideline 407
Species/strain	:	Wistar Hanlbm (SPF) rat
Group Size	:	10 rats per sex : control and the high dose, 5 rats per sex : mid and low doses
Test material	:	MIP 2985
Batch no	:	CGF-F016740/0018
Purity	:	98%
Dose	:	15, 50 and 150 mg/kg bw
Treatment Period	:	28 days
Observ. period	:	14 days
GLP	:	in compliance

MIP 2985 was administered in feed for 28 days at theoretical dose levels of 15, 50 and 150 mg/kg body weight/day while the control group received the normal diet. The corresponding effective daily intake for males was 12.25, 39.59 and 135.46 mg/kg body weight, and for females, 13.16, 40.50 and 132.54 mg/kg body weight for low, mid and high dose groups respectively. A 14-day recovery period was allowed for 5 animals per sex in the control and high dose groups.

The animals were examined for clinical signs daily and checked twice daily for mortality. Food consumption and body weight were recorded once pre-test, and weekly thereafter and body weight at necropsy. A functional observational battery (modified Irwin screen test), grip strength and locomotor activity were performed during week 4. Urine and blood for haematology and clinical biochemistry were collected from all animals,. All animals were killed and descriptions of all macroscopic abnormalities were recorded. The major organ weights (absolute and relative) were recorded on the date of necropsy. Samples of major organs from control and top dose groups, as well as liver and thyroid glands and all gross lesions from all animals were examined by light microscopy. Only liver, thyroid gland and gross lesions were examined microscopically from rats of mid and low dose groups.

All animals survived during the study. No quantitative or qualitative differences of clinical parameters from the control values were noted. A dark discoloration of the faeces was observed in all animals of the high-dose group on Day 3 dosing to Day 3 recovery. In the high dose group, urine of 9 males and 3 females was a deep yellow colour after 4 weeks but by week 6, the urine colour was normal. A lack of appetite was observed in females of the high-dose group only during the treatment period. The relative food consumption was similar in all groups. Mean body weight and body weight gain were slightly increased in the mid and low dose group males, and reduced in high-dose group females when compared with the control. There were no dose-related effects in the functional observational battery, grip strength measurement, and locomotor activity.

Minor clinical laboratory changes were recorded after 4 weeks treatment between the high dose group and the controls. At the high dose, there was a slight increase in circulating lymphocytes (relative) as well as a slight decrease in segmented neutrophils (relative and/or absolute) in both sexes ($p<0.05$). At end of the treatment-free recovery period, these haematological parameters were found to be similar to the controls, indicating reversibility. Clinical biochemistry results showed the following findings in the high dose group :

Total cholesterol level slightly increased in both males and females ($p<0.01$)

Triglyceride level slightly increased in both males ($p<0.01$) and females ($p<0.05$); a slight increase was also recorded in the mid-dose group females ($p<0.05$)
Phospholipid level slightly increased in both males and females ($p<0.0l$)
Albumin level slightly increased in both males ($p<0.05$) and females ($p<0.0l$)
Globulin level slightly reduced in both males ($p<0.05$) and females (statistically not significant)
Albumin to globulin ratio slightly increased in both males and females ($p<0.05$).

At the end of the treatment-free recovery period, there was an indication of reversibility for most parameters. However, slightly higher significant ($p<0.05$) values were still observed in the triglyceride, phospholipid and albumin concentrations in male rats. The authors considered these findings are considered to primarily reflect adaptive changes in lipid metabolism. Histopathological correlates to these hepatic metabolic changes were not found. Urinalysis parameters were not adversely affected by treatment and only the reversible deep-yellow urine discoloration was considered test article-related. Organ weights and organ to body weight ratios were higher in the mid-dose group males, but were attributed to the higher terminal body weights of the rats. Macroscopically, there was a reddish brown discoloration of the thyroid gland observed at 4 weeks in 80% of the males and in all the females of the high-dose group, 40% of the males in mid-dose group and one control female. At 6 weeks, all the high dose recovery group animals showed thyroid discoloration. All other lesions recorded were considered to be within the normal range of background findings commonly seen in rats of this strain and age.

Based on these results, the NOAEL of MIP 2985 was estimated as 12.25 mg/kg body weight/day.

Ref : 4

2.3.5. Repeated dose dermal toxicity

Guideline	:	OECD Guideline 402
Species/strain	:	Albino Guinea pig, Ibm: GOHI, SPF
Group Size	:	4 males and 4 females
Test material	:	MIP 2985
Batch no	:	CGF-F016740/0018
Purity	:	98%
Dose	:	1.0, 0.5, 0.1%w/w
Observ. period	:	14 days
GLP	:	in compliance

A 14-day repeated dose dermal toxicity study to assess the cumulative irritation potential with MIP 2985 was applied daily at concentrations of 1.0, 0.5 and 0.1% w/w in double-distilled water. The skin was shaved prior to the study and 2 circular areas 7cm^2 were marked. The animals were shaved four times during the first week and 3 times in the second week. A depilatory cream was used on Day 15 after the final application. One male and female served as controls and received double-distilled water. No grading scores were recorded from test day 2-14, as no depilation was used during this period.
However after depilation on test day 15, no skin reaction was observed. These data were confirmed by histopathology. Under the experimental conditions, the study authors considered MIP 2985 to be a non-irritant when repeatedly applied to guinea pigs skin.

Ref : 3

2.3.6. Repeated dose inhalation toxicity

No data

2.3.7. Sub-chronic oral toxicity

Guideline	:	OECD 408 (1981) and Directive 96/54/EEC
Species/strain	:	Wistar rat, Hannover (SPF)
Group Size	:	10 males + 10 females per dose
Test material	:	MIP 2985
Batch no	:	CGF-F016740/0018
Purity	:	98%
Dose	:	0, 10, 50 and 250 mg/kg bw/day
Exposure period	:	13 weeks
GLP	:	in compliance

MIP 2985 was administered in feed at theoretical dose levels of 10, 50 and 250 mg/kg bw/day while the control group received the normal diet. The corresponding effective daily intake, based on food consumption and body weight for males was 9.8, 49.5 and 253.4 mg/kg body weight, and for females 10.1 51.2 and 247.3 mg/kg. bw for low, mid and high dose groups respectively.

The animals were examined for clinical signs daily and checked twice daily for mortality/viability. Food consumption and body weight were recorded once pre-test, and weekly thereafter and body weight at necropsy. Ophthalmoscopic examination was performed at pre-test and at week 13 (control and high-dose animals). A functional observational battery (modified Irwin screen test) was performed during pre-test and at week 12 on all rats and grip strength and locomotor activity were evaluated. At week 13, blood samples were collected for haematology and clinical biochemistry from all animals and urine samples were collected for analysis. After 13 weeks, all animals were weighed and killed and descriptions of all macroscopic abnormalities were recorded. The major tissues and organ were collected from all animals and absolute and relative weights were recorded at necropsy for adrenals, brain, heart, kidneys, liver, ovaries, spleen, testes, thyroid, and thymus. Samples of major organs from control and high-dose and all gross lesions from all animals were processed as haematoxylin-eosin slides and examined by light microscopy.

Deaths did not occur during the study. No clinical signs considered related to the test substance occurred. In the high dose group, a reduction in mean food consumption was 25% in males and 20% in females. An 8% reduction in the males of the mid dose group was noted. These data were considered test substance-related.

No effect of MIP 2985 was observed in any group on food consumption relative to body weight. The body weight and body weight gain in males and females of the high-dose group showed a significant ($p<0.05$) reduction. In the mid-dose group males, a non-significant reduction was observed. The low dose group was not affected.

Ophthalmologic findings were noted in a small proportion of animals from all groups during the study. The following findings, considered unrelated to treatment, occurred at similar incidences in the high and in the control group: persistent pupillary membranes, comeal opacities, anterior synechia and persistent hyaloid vessels in the vitreous body. From the functional observational battery, grip strength measurement and locomotor activity, some incidental findings were recorded: decreased pupil diameter and contraction response in one male of the high-dose group, increased aggressiveness and fearfulness in females of the high-dose group; a statistically significant decreased grip strength of both forelimb and hind limb in group 4 was attributed to the reduced body weight of the animals.

Haematology results showed a significant ($p<0.05$) increase in methaemoglobin levels in males and females of the high dose group. A dose-related decrease of the number of white blood cells ($p<0.01$) was noted in high dose males along with a significantly ($p<0.01$) reduced absolute number of lymphocytes; these changes were considered dose-related. The clinical biochemistry parameters showed a significant ($p<0.01$) increase of gamma-glutamyl transferase levels in rats of the high dose group (212 % and 202 % in males and females, respectively). The high dose group animals also showed significant ($p<0.01$) decreases in creatinine levels, in amount of total protein accompanied by disproteinemia, and in urea and uric acid levels. Glucose, total bilirubin and phospholipids levels were decreased in high dose males; total cholesterol, creatine kinase and alkaline phosphatase were increased in high dose females. The authors considered these findings to be metabolic adaptations to MIP 2985 and of no toxicological relevance. No abnormalities were revealed by urinalysis results.

Decreased ($p<0.05$) absolute organ weights and/or organ weight ratios of most organs were recorded for males in the mid and high dose groups and for females in the high dose group. Macroscopically, there was a reddish discoloration of the forestomach mucosa, the thymus was reduced in size (high dose), and the thyroid gland was discoloured red-brown (mid dose) or black (high dose). The microscopic findings occurred mainly in the high dose group and included the following: Minimal to slight diffuse hepatocellular hypertrophy associated with moderately increased incidence of focal necrosis, and decreased haematopoiesis. The spleen showed a decreased incidence of increased haematopoiesis. In the kidneys an increased incidence of intratubular granular casts and transitional cell hyperplasia occurred in females. Non-glandular stomach dilation was observed in one male and one female in the high dose group and most of the high dose animals showed congestion; a low incidence of glandular stomach erosion also occurred. In the thymus, the observed cortical atrophy was considered as a condition of stress rather than an immunotoxic effect. The ovaries showed an interstitial cell hyperplasia considered as a non-neoplastic proliferation of cellular components of the interstitial gland. An increased diffuse fatty change was observed in the adrenal cortices of males and females of the high dose group. Thyroid of mid- and high-dose males and females showed an increased incidence of follicular cell hypertrophy, pigmented colloid, and pigmented follicular cells. The pituitary gland of males of these two groups showed an increased incidence of TSH/ACTH cell hypertrophy.
The study authors estimated the NOAEL to be 50 mg/kg body weight/day, and the NOEL, 10 mg/kg/day, the lowest dose used. The SCCNFP concluded that the NOAEL should be 10 mg/kg bw/day in light of the effects on the thyroid and pituitary.

Ref : 5

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	:	OECD 404 (1981)
Species/strain	:	albino rabbits, New Zealand
Group size	:	1 male, 2 females
Test substance	:	MIP 2985
Batch number	:	029753A8AA
Purity	:	97.2% by UV-VIS ; 98.8% by HPLC
Dose	:	0.5g applied to 6.25 cm^2 of intact skin
GLP	:	In compliance

After clipping the back and flanks, 0.5g of the test material was applied to an area of approximately 6.25 cm^2 under a semi-occlusive dressing. The patches were removed after 4 hours and observations made at 0.5, 1, 24, 48 and 72 hours after removal.

Results
No erythema or oedema was observed. The primary irritation index was 0.0. The test material was considered to be non-irritating to the rabbit skin.

Ref. : 6

2.4.2. Irritation (mucous membranes)

Guideline	:	OECD 405 (1981)
Species/strain	:	albino rabbits, New Zealand
Group size	:	3 (sex not stated)
Test substance	:	MIP 2985
Batch number	:	029753A8AA
Purity	:	97.2% by UV-VIS; 98.8% by HPLC
Dose	:	0.0360 g in 0.1ml
GLP	:	In compliance

The test material was applied instilled into the lower lid of the right eye of each animal. The left eye served as the untreated control. The eyes of the 3 animals remained unrinsed for approximately 24 hours after instillation of the test material.
1, 24, 48, 72 and 96 hours and 7, 14 and 21 days after instillation of the test material, the treated eyes of the rabbits were observed for signs of ocular irritation. Corneal injury was assessed using sodium fluorescein (followed by a saline wash) on all animals at 24 hours post-instillation.

Results
There were no effects involving the cornea or iris.
Redness of the conjunctiva was noted in all animals from 1 hour to 14 days post instillation and in 1 animal at 21 days post instillation. Chemosis was noted in 2 animals from 1 hour through to 7 days postinstillation and in 1 animal on days 14 and 21. Discharge was noted in 2 animals from 1 hour to day 14 and in 1 animal through to day 21. There was no evidence of corrosion.
A maximum mean score of 7.3 at 1 and 24 hours post instillation was determined. MIP 2985 was moderately irritating to the eyes of the rabbits under the conditions of the study.

Ref. : 7

2.5. Sensitisation

Magnusson and Kligman Guinea pig maximisation test

Guideline	:	OECD 406		
Species/strain	:	albino guinea pigs		
Group size	:	15 females (10 test and 5 control)		
Test substance	:	MIP 2985		
Batch number	:	CGF-F016740/0018		
Purity	:	>98%		
Dose	:	Intradermal induction	:	A 5% aqueous solution with and without Freund's Complete Adjuvant.
		Topical induction	:	A 50% preparation of test material under occlusion for 48 hours. Controls received vehicle only.
		Challenge	:	14 days later by exposing 25% aqueous dilution of the test substance (24 hours, occlusion).
GLP	:	In compliance		

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

Results
None of the animals of the control or test group were observed with skin reactions after challenge with a non-irritating preparation of 25% of the test material. MIP 2985 was considered not to be a sensitizer under the test conditions.

Ref. : 8

2.6. Teratogenicity

Guideline	:	OECD 414
Species/strain	:	Wistar rat, Hanlbm (SPF)
Group Size	:	22 mated females per dose
Test material	:	MIP 2985
Batch no	:	CGF-F016740/0018
Purity	:	98%
Dose	:	0, 20, 60 and 180 mg/kg bw/day
Treatment period	:	Days 6 to 17 post coitum,
GLP	:	in compliance

The animals were dosed with10ml /kg by gavage once daily. The control group received only the vehicle (double distilled water).

Food consumption was recorded for the following periods: days 0-6, 6-12, 12-18 and 18-21 post coitum; body weight was recorded daily from day 0 until day 21 post coitum. Clinical observations and mortality were recorded at least twice daily. At post mortem, on day 21, necropsy, all internal organs were examined with emphasis on the uterus, uterine contents, position of foetuses in the uterus and number of corpora lutea. The uteri of all females with live foetuses were weighed at

necropsy on day 21 post coitum; the foetuses were removed from the uterus, weighed, sexed, and examined for gross external abnormalities.

Maternal deaths did not occur during the study and clinical signs of toxicity or reactions to treatment did not occur in any group. A dose-dependent reduction of the food consumption was observed during the treatment period in the mid- and high-dose groups (-7.6 % and -23.5 % respectively); an increase of + 5.5 % was observed in the high dose group after the treatment period. The mean body weight gain was reduced only in the high dose group, these data being correlated with the decreased food consumption. Mean post-implantation loss and mean number of foetuses per dam were similar between treated and control dams in the low- and mid-dose groups. The increased post-implantation loss observed only in the mid dose group was considered to be incidental. No abnormal findings were noted in any female of any treated group.

The mean foetal body weights were similar in all groups except for a slight increase observed in the mid-dose group, which was attributed to the slightly reduced mean number of foetuses per dam. The sex ratio for foetuses was similar in all groups. Some abnormal findings were noted during foetal examination: externally, one cleft palate was observed in the low dose group. Skeletal changes included a small number of foetuses in each group with abnormally shaped sternebrae. These were not considered related to the test substance, as they were within the range for historical controls.

Under the experimental conditions, MIP 2985 was not toxic to embryo or foetus and was not teratogenic. The study authors considered the NOEL for the maternal effects is 20 mg/kg bw/day and for foetal effects the NOEL is 180 mg/kg bw/day.

Ref. : 11

2.7. Toxicokinetics (including Percutaneous Absorption)

In vitro study of percutaneous absorption

Guideline	:	/
Tissue	:	Human epidermal skin membrane (exposure area: 1 cm^2)
Method	:	Franz diffusion cells
Test material	:	Basic Red 51, 0.214% in a hair dye formulation
Batch No	:	CGF-F016740/0018 (Purity: 98.6%)
Dose level	:	103 mg/cm^2 of the formulation
Receptor fluid	:	25% (w/v) ethanol in PBS (pH 7.4)
Replicate cells	:	12
Analyt method	:	HPLC (detection at 523 nm). Detection limit: 1 ng/ml.
GLP	:	In compliance

The skin penetration of Basic Red 51 was evaluated in a static Franz diffusion cell system using epidermal membranes obtained from full-thickness human female skin. The integrity of the skin was checked by ^3H_2O (limit of permeability coefficient: $1.5 * 10^{-3}$ cm/h). The solubility of the dye in the receptor fluid was higher than 200 µg/ml.
The dye formulation (103 mg/cm^2) equivalent to 221 µg/cm^2 of the dye active principle was applied on the skin surface for 30 min. Then, the skin surface excess was washed off with warm water (40ºC) and left unoccluded for the entire 48 hour exposure period. After 48 hours, the dye content was determined in the following compartments: skin surface excess (washings, wipes and donor chamber), SC, epidermis and receptor fluid.

Results
Under the present experimental conditions, a total recovery of the dye of 98.9% has been obtained.
Most of the hair dye applied on the skin surface was removed with the washing procedure (98.8 % or 221.5 µg/cm²). The content of the dye detected in the SC was: 0.01 % (0.031 µg/cm²). A total of 0.018% of the applied dose is reported to have penetrated into the epidermis and permeated into the receptor fluid during 48 h. This corresponds to a percutaneous absorption of 0.040 µg/cm².

Comment
The substance was not tested in the presence of an oxidising agent and the applied dose of 103 mg/cm² is higher than the amount recommended by the SCCNFP (20 mg/cm²).

Ref. : 12

2.8. Mutagenicity/Genotoxicity

2.8.1 Mutagenicity/Genotoxicity *in vitro*

Bacterial Reverse Mutation Test

Guideline	:	OECD 471
Species/strain	:	*S. typhimurium,* TA98, TA100, TA1535, TA1537, *E. coli* WP2 uvrA
Replicates	:	Triplicate plates, 2 independent tests
Test substance	:	MIP 2985
Batch no	:	029753A8AA, purity : 98.8 % by HPLC
Concentrations	:	*S. typhimurium* Without metabolic activation Test #1 : 3.33 – 333 µg/plate (6 doses) With metabolic activation (rat liver) Test #1 : 3.33 – 500 µg/plate (6 doses) With reductive metabolic activation (hamster liver) Test #1 : 3.33 – 500 µg/plate (6 doses) *E. coli* With and without metabolic activation (normal & reductive systems) Test #1 : 3.33 – 333 µg/plate (6 doses) *S. typhimurium* Without metabolic activation Test #2 : 3.33 – 200 µg/plate (6 doses) (the last 2 doses are toxic) With metabolic activation (rat liver) Test #2 : 3.33 – 333 µg/plate (6 doses) With reductive metabolic activation (hamster liver) Test #2 : 10 – 500 µg/plate (6 doses) *E. coli* Without metabolic activation (normal & reductive systems) Test #2 : 3.33 – 333 µg/plate (6 doses) (the last 2 doses are toxic) With metabolic activation (normal & reductive systems)

Test #2 : 3.33 – 333 μg/plate (6 doses)) (the last doses were toxic)
GLP : In compliance

MIP 2985 has been investigated for gene mutation in *Salmonella typhimurium and E. coli* using the direct plate incorporation method both with or without S9 mix.
S9 mix from different origin have been used : Standard : Sprague-Dawley rats injected i.p. with Aroclor™ 1254 ; Reductive : uninduced male Golden Syrian hamsters.
Negative and positive controls were in accordance with the OECD guideline.

Results

Test # 1
In the absence of activation, no dose related and biologically relevant increase in revertant numbers was observed, in all but one tester strains (Salmonella TA 98). The increase is according to the OECD criteria.
In the presence of rat (commonly used S9) activation : No dose related and biologically relevant increase in revertant numbers was observed, in any of the tester strains used (*Salmonella or E. coli*).
In the presence of Hamster (reductive S9) activation : an increase in revertant numbers was observed for TA 98 - a frameshift tester strain - at the dose of 100 μg/plate. There is a trend for a dose relationship until cytotoxicity that could have prevented the expression.
For the other strains (including E. coli), no statistically or biologically relevant increase of mutant frequencies have been observed as compared to the controls.
Positive controls showed the expected response.

Test # 2
In the absence of activation, no dose related and biologically relevant increase in revertant numbers was observed, in any of the tester strains.
In the presence of rat activation, no dose related and biologically relevant increase in revertant numbers was observed, in any of the tester strains.
In the presence of Hamster (reductive S9) activation, a statistical and dose related significant increase in revertant numbers was observed for TA 98.

Conclusions
The test is acceptable for evaluation.
Based on the reversion rate, and under the conditions of the 2 assays performed, it could be concluded that the test agent Basic Red 51, in the presence of reductive S9 mix, shows clear evidence of mutagenic activity in tester strain TA 98. Such positive results may be the consequence of the metabolizing properties (azo-reduction) of the S9 mix fraction from hamster. The higher amount of aromatic amines released, which are metabolised to electrophilic molecules that may react with DNA, might explain the positive results observed in TA 98.

Ref. : 13

In Vitro Mammalian Chromosomal Aberration Test

Guideline : OECD 473
Species/strain : Human lymphocytes (pooled cultured blood samples)
Replicates : Duplicate cultures, 2 independent experiments
Test substance : MIP 2985 in cell culture grade water

Batch no	:	029753A8AA, purity : 98.8% by HPLC
Concentrations	:	Test #1 3 h without S9 : 20.2, 28.8, 41.2 µg/ml 3 h with S9 : 41.2, 58.8, 84, 120 µg/ml Test # 2 3 h with S9 : 22.5, 45, 90, 120 µg/ml 22.2 h without S9 : 17.5, 22.5, 30, 35 µg/ml
GLP	:	In compliance

MIP 2985 has been investigated for induction of chromosomal aberrations in human pooled lymphocytes. The test concentrations were established from a preliminary toxicity study. Liver S9 fraction from Aroclor 1254-induced rats was used as the exogenous metabolic activation system.

Results
pH & Top dose concentration
pH was not significantly changed at the maximum dose tested of 4990 µg/ml(pH =7.5).
Molarity for the maximum dose tested in the assay (2990 µg/ml) was found to exceed slightly the maximum value recommended (10 mM) (10 mM = 2798 µg/ml : molecular weight 279.8). However, the small deviation (2990-2798 = 192) is devoid of biological relevance.

Structural chromosome aberrations

With or Without S9 mix :
No statistically or biologically significant increase in the number of aberrant cells was observed as compared to the corresponding solvent control.

Polyploidy
No significant increase of aneuploidy and/or endoreduplicated cells was noted.

Conclusions
The assay is acceptable for evaluation.
Basic Red 51 is considered negative for clastogenic and/or aneugenic activity in human lymphocytes in the presence or the absence of activation under the conditions of the test.
To be noticed: the tests have been performed only with the Standard metabolic activation system (rats injected i.p. with Aroclor™ 1254).
No definitive conclusions can be made at present.

Ref. : 14

In Vitro Mammalian Cell Gene Mutation Test

Guideline	:	OECD 476
Cells	:	Chinese Hamster V-79 cell line (mutation at the HPRT locus)
Replicates	:	2 independent tests
Test substance	:	MIP 2985 in serum free medium
Batch no	:	11R-1, purity : 60 %
Concentrations	:	Test #1 Without metabolic activation : 3 – 300 µg/ml (6 doses) With metabolic activation : 3 – 400 µg/ml (6 doses)

Test #2
Without metabolic activation : 3 – 150 µg/ml (6 doses)
With metabolic activation : 3 – 200 µg/ml (6 doses)

GLP : In compliance

MIP 2985 in serum free medium (batch KS11R-11823) has been investigated for gene mutation at the HGPRT locus in V79 Chinese hamster cell line.
S9 mix from different origin have been used : Standard : Sprague-Dawley rats injected i.p. with Aroclor™ 1254 in the second test; Reductive : uninduced male Golden Syrian hamsters in the first test.

Results
Toxicity
Toxicity occurred at dose of 100 µg/ml and above with and without activation in both assays.

Precipitate
No visible precipitation was observed.

Mutant frequency
Test # 1
No statistically or biologically significant increase in mutant frequency was observed over the concurrent solvent controls for any doses in all conditions.

Test # 2
Without S9 mix, the negative control value was extremely low (1.4 per million cells). The treated doses showed an increase in mutation frequency without dose dependence. Therefore the increases observed are thought to be devoid of biological relevance.
With S9 mix : No statistically or biologically significant increase in mutant frequency was observed over the concurrent solvent controls for any doses.

Conclusions
The test is acceptable for evaluation.
MIP 2985 did not demonstrate mutagenic potential on the HPRT gene of V79 cells.

Ref. : 15

2.8.2 Mutagenicity/Genotoxicity *in vivo*

Mammalian Erythrocyte Micronucleus Test

Guideline : OECD 474 (1983)
Species : NMRI mice
Group sizes : 6 male and 6 female
Material : MIP 2985
Batch no : 0017, purity > 88 %
Dose levels : MIP 2985 was administered by 1 single oral dose of :
10, 33 and 100 mg/kg bw for the 24 h sacrifice time
100 mg/kg bw for the 48 h sacrifice time.
GLP : In compliance

MIP 2985 has been investigated for induction of micronuclei in the bone marrow cells of male or female mice. Dose levels were determined by a preliminary range finding study in which observable toxic effects were seen at doses of 1000 and 2000 mg/kg bw. The substance was administered by a single intragastric gavage and the groups of animals sacrificed 24, 48 and 72 hours after administration. Negative and positive controls were in accordance with the OECD guideline.

Results
Number of cells scored : a total of at least 1000 erythrocytes were examined from each animal ; the incidence of micronucleated erythrocytes and the ratio of polychromatic erythrocytes to normochromatic erythrocytes were calculated.

Results
NCE : the mean number of NCE (mature differentiated cells) was not significantly increased after treatment as compared to controls; this reflects the lack of cytotoxicity of the test agent.
PCE 24 h 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.
PCE 48 h sampling time : no statistically significant or biologically relevant increase in the incidence of micronucleated polychromatic erythrocytes over the concurrent vehicle control values were observed.

Conclusions
Under the conditions of the test it can be concluded that MIP 2985, at doses at which some signs of clinical toxicity were recorded, does not induce statistically significant increase in the frequency of micronucleated PCE.
Therefore, MIP 2985 is not clastogenic and/or aneugenic in this mouse bone marrow micronucleus test. However, in the absence of a toxicokinetics study we may not assume that the test agent has reached the target cells.

Ref. : 17

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

Guideline	:	OECD draft guideline 486
Species/strain	:	Wistar rat, HanIbm: WIST (SPF) strain
Group size	:	4 male rats
Test substance	:	MIP 2985
Batch no	:	CGF-F016740/0018 (purity > 98 %)
Dose levels	:	0, 75 and 300 mg/kg bw, by single oral gavage
Exposure time	:	16 hours: all dose groups; 2 h: high dose group
GLP	:	In compliance

MIP 2985 has been investigated for induction of unscheduled DNA synthesis in rats hepatocytes at 2 doses 75 and 300 mg/kg bw.
Positive controls were in accordance with OECD guideline for 60 hours treatment and UDS analysed by autoradiography. 3 males were used per dose/time sampling.

Results
The viability of the hepatocytes was not substantially affected by the treatments.

Treatment with MIP 2985 at doses of 75 & 300 mg/kg yielded group mean NNG values less than 0 for both experiment time and caused no significant increases, as compared to control, in the mean nuclear grain counts.

Conclusions
According to OECD guidelines, the study is inadequate because of the lack of a positive control for the short time exposure.

Ref. : 16

2.9. Carcinogenicity

No data

2.10. Special investigations

Photoirritation

Guideline	:	OECD draft (1995) "Acute dermal photoirritation dose-response test"
Species/strain	:	Himalayan spotted guinea pigs
Group size	:	15 males (10 test and 5 control)
Test substance	:	MIP 2985
Batch number	:	CGF-F016740/0018
Purity	:	> 98%
Dose	:	0.025 ml/2cm^2 of 50%, 25%, 15% and 10% aqueous dilutions. Skin at test sites treated 30 minutes before application with 2% DMSO in ethanol.
GLP	:	In compliance

MIP 2985 was applied epicutaneously to skin areas of 2 cm^2 on both flanks. 30 minutes after application of the test materials, the left flank was exposed to 20J/cm^2 UVA. The right flank remained unexposed to light after treatment and served as a reference site. Control animals were exposed to UVA similarly but treated with solvent only. Cutaneous reactions were evaluated at 24, 48 and 72 hours after application.

Results
There were positive reactions in all animals at the sites treated with 50% of the test material, both with and without UVA. The reactions were considered to be an irritant effect unrelated to UVA exposure. There were no other reactions and it was concluded that under the test conditions, MIP 2985 does not exhibit a phototoxic potential in the guinea pigs of the strain and age.

Ref. : 9

Photoallergy

Guideline	:	CTFA Safety Testing Guideline
Species/strain	:	Himalayan spotted guinea pigs
Group size	:	30 males (20 test and 10 control)
Test substance	:	MIP 2985
Batch number	:	CGF-F016740/0018

Purity	:	>98%		
Dose	:	Induction	:	nuchal skin of the test group shaved. Test site of 6-8 cm^2 defined by four 0.1ml intradermal injections of Freund's Complete Adjuvant and physiological saline 1:1 into the corners. 0.1ml of 50% MIP 2985 applied to area of 8 cm^2. The site was then exposed to 1.8J/cm^2 UVB and 10J/cm^2 UVA. The application and irradiation (after shaving) was repeated on days 3, 6, 8, and 10.
		Challenge	:	3 weeks after the start of the induction procedure, test sites of 2cm^2 were marked and 0.025 ml/2cm^2 of 50%, 25%, 15% and 10% were applied to the left flank and then irradiated with 10J/cm^2 UVA. After irradiation of the left flank, the right flank was treated with the test materials without irradiation.
GLP	:	In compliance		

3 hours prior to the first readings, the application sites were depilated with a depilatory cream (VEET cream). Each animal was assessed for reactions at 24, 48 and 72 hours after challenge.

Results
A very slight red discoloration produced by the test material at the application sites were observed from test days 2 to 22. No skin reactions were observed in the test animals treated with 50% test material during the induction phase. No reactions were observed on the irradiated or non-irradiated flanks of the control and test animals treated with 50, 25, 15 and 10% test material. The data indicated that MIP 2985 does not exhibit photoallergic potential.

Ref. : 10

2.11. Safety evaluation

Not applicable

2.12. Conclusions

Chemical identification of an impurity in the test material, content up to 0.4%, has not been performed. No experimental data is provided on stability of the test material.

The acute oral LD_{5O} was set at 250 - 500 mg/kg bw in females and at 500 - 1000 mg/kg bw in males. The acute dermal LD_{5O} is greater than 2000 mg/kg bw.
The NOAEL was set at 12.25 mg/kg bw/day (repeated dose oral toxicity study). In light of the effects on the thyroid and pituitary (sub-chronic oral toxicity study), the NOAEL was set at 10 mg/kg bw/day. Basic Red 51 was not toxic to embryo or foetus and was not teratogenic. The NOEL for the maternal effects was set at 20 mg/kg bw/day and at 180 mg/kg bw/day for foetal effects.

Basic Red 51was not irritating to the skin and moderately irritating to the eyes. It is not considered to be a sensitiser.

A total of 0.018% of the applied dose is reported to have penetrated, corresponding to a percutaneous absorption of 0.040 µg/cm². However, the substance was not tested in the presence of an oxidising agent.

Basic Red 51 has been tested in prokaryotic and mammalian cells for gene mutation, and in mammalian cells for chromosomal aberration in vitro. Two *in vivo* tests have been performed (bone marrow micronucleus and UDS tests). The *in vitro* test for gene mutation in prokaryotes has been found positive in the presence of a reducing metabolic activation system.
The *in vitro* test for gene mutation in mammalian cells showed that the test agent is non mutagenic under both activation conditions.
The *in vitro* test for clastogenicity in human lymphocytes is negative, with only a normal activation system.
The *in vivo* micronucleus test in mice gave negative results; no firm evidence that the bone marrow was reached by the test agent was noted.
The *in vivo/in vitro* UDS on rats hepatocytes is negative for the treatment of 16 hours; the effect of 2 hours treatment could not be evaluated due to the absence of a concurrent positive control. This is why the study is considered as inadequate according to the OECD guideline 486.
Considering that the metabolic behaviour suspected in the strain TA 98, could have influenced specifically the results observed so far, and considering the minor inadequacy of the UDS test and the absence of toxicokinetics data in the *in vivo* micronucleus assay, it may be concluded that there are insufficient data to evaluate the mutagenic potential of this dye.

2.13. References

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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 :

* information on the stability of the test material in the test preparations and in the hair dye formulations.

* percutaneous absorption study in accordance with the SCCNFP Notes of Guidance, if used in an oxidising environment.

* 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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