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

CONCERNING

6-NITRO-O-TOLUIDINE

COLIPA n° B56

adopted by the SCCNFP during the 24th plenary meeting
of 24-25 June 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 6-Nitro-o-toluidine 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

6-nitro-o-toluidine (INCI name)

2.1.2. Chemical names

Chemical name : 1-Amino-2-methyl-6-nitrobenzene
CAS name : Benzenamine, 2-methyl-6-nitro
Synonyms : 2-Amino-3-nitrotoluene; 2-methyl-6-nitro-benzenamine; 2-methyl-6-nitroaniline; 2-methyl-6-nitrophenylamine; 2-nitro-6-methylaniline; 3-nitro-2-amino-toluene; 3-nitro-2-amino-toluol; 3-nitro-2-toluidine; 6-methyl-2-nitroaniline; o-toluidine, 6-nitro-

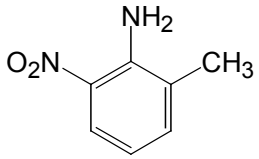
2.1.3. Trade names and abbreviations

Trade name : IMEXINE® FP (Chimex)
COLIPA n° : B56

2.1.4. CAS no./EINECS n°

CAS no : 570-24-1
EINECS n° : 209-329-3

2.1.5. Structural formula



2.1.6. Empirical formula

Emp. Formula : $C_7H_8N_2O_2$
Mol weight : 152

2.1.7. Purity, composition and substance codes

All analytical data relate to batch 9060122.

Purity	:	> 99% (titre as determined by HPLC)
Water content	:	0.04%
Ash content	:	< 0.1%
Heavy metals	:	< 10 ppm

Potential impurities

- Reagents and intermediate reaction products

o-toluidine	:	< 100 ppm
2-methyl-benzene-1,6-diamine	:	< 100 ppm
3-nitro-o-toluidine	:	< 100 ppm
4-nitro-o-toluidine	:	475 ppm
5-nitro-o-toluidine	:	< 100 ppm

- Solvent residues

Ethanol	:	250 ppm
Chloride ions	:	0.02%

2.1.8. Physical properties

Appearance	:	dark red crystalline powder
Melting point	:	94.5-95 °C
Boiling point	:	/
Flash point	:	100 °C
Density	:	840 g/l
Rel. vap. dens.	:	/
Vapour Press.	:	/
Log P_{ow}	:	2.2 at pH 7.2, calculated (Software CLOGP3.64)
Storage	:	Well closed, protected from light

2.1.9. Solubility

Water (25°C)	:	insoluble
96% ethanol	:	1%
Ethylglycol	:	10%
Chloroform	:	10%
Receptor fluid	:	40 mg/l

* receptor fluid for percutaneous absorption study : 0.9% aqueous sodium chloride

General comments on analytical and physico-chemical characterisation

* Impurities have been detected in test material at 100 ppm (detection limit).

* o-Toluidine and 2-methyl-benzene-1,6-diamine are classified carcinogenic category 2 according to Directive 67/548/EEC.

* A loss of 6 % of the test material was noted in the 0.7 % formulation used in the percutaneous absorption study. It was stored at 45 °C for 1 month and a further month at room temperature (range not specified). There is no description of the degradation products.

2.2. Function and uses

6-Nitro-o-toluidine is intended to be used in semi-permanent hair dye formulations at a maximum concentration of 0.35 %.

TOXICOLOGICAL CHARACTERISATION

2.3. Toxicity

2.3.1. Acute oral toxicity

Guideline	:	OECD 401 (1987)
Species/strain	:	Sprague Dawley rat ICO OFA-SD (IOPS Caw)
Group size	:	5 males + 5 females
Test substance	:	IMEXINE FP suspended in 1,2-propanediol
Batch no	:	9060122
Purity	:	> 99 %
Dose	:	2000 mg/kg bw
Observation Period	:	14 days
GLP	:	in compliance

A single limit dose of test substance by gastric gavage was given. The animals were observed for clinical signs and mortalities for 14 days. Bodyweights were recorded at intervals. Macroscopic abnormalities were recorded at autopsy.

Results
There were 3 mortalities (1 male, 2 females) on day 1 post-dosing. Clinical signs including sedation, hypokinesia and dyspnea were seen in most animals post dosing. All animals appeared normal by day 4. Weight gain of surviving animals was normal for the strain. The oral LD50 was reported to be greater than 2000 mg/kg bw.

Ref. : 1

2.3.2. Acute dermal toxicity

No data

2.3.3. Acute inhalation toxicity

No data

2.3.4. Repeated dose oral toxicity

High dose rat study

Guideline	:	OECD 407 (1981)
Species/strain	:	Sprague Dawley Crl: CD (SD) BR
Group size	:	10 males + 10 females
Test substance	:	IMEXINE FP suspended and homogenised in 0.5% methylcellulose hydrogel
Batch no	:	9060122
Purity	:	> 99 %
Dose levels	:	0, 20, 100 and 500 mg/kg bw/day, 7 days/week by gavage
Exposure period	:	29-30 days
GLP	:	in compliance

Groups of 10 male and 10 female rats were dosed with the test substance by gavage at 0, 20, 100 and 500 mg/kg bw/day, 7 days/week for 29 or 30 days, depending on autopsy date. The dosing solutions were analysed during weeks 1 and 4 for stability and verification of homogeneity and concentration, but the results are not included in the report. During the study, clinical signs and mortality were monitored daily and bodyweight and food consumption weekly. During week 4, blood was sampled from the orbital sinus for haematology and blood biochemistry; urine was collected overnight for urinalysis. At the end of the treatment periods, a full autopsy was conducted, recording weights, macroscopic and microscopic examination of major organs (microscopic examination was only conducted for tissues of mid and low dose if macroscopic findings were reported). Ophthalmoscopy was conducted before the start of the study and at the end of the treatment period on all animals.

Results
There were no mortalities. Clinical signs of treatment were a decrease in spontaneous activity on days 1 and 2 post-dosing, and hypersalivation from day 9 post-dosing, affecting most animals treated at 500 mg/kg bw/day. Decreased spontaneous activity and dyspnea was also observed in one female treated at 500 mg/kg bw/day. All high dose animals exhibited orange-coloured urine, which was attributed to the staining properties of the substance.
Food consumption was comparable for all groups. Decreased bodyweight gain was noted in both sexes at the high dose compared with control. By week 5, this was significant, (females, 90%, males, 86% of control) The males lost weight in this final week. Ophthalmologic examinations revealed corneal dystrophy, in one male at 20 mg/kg bw/day, one male at 100 mg/kg bw/day and 2 males and 4 females at 500 mg/kg bw/day. Because of the dose-response relationship the study author considered that this was probably treatment-related.
Minor changes were reported in some haematological parameters but all individual values were within the range of historical controls and the study author concluded that the changes were not of toxicological significance. Alanine aminotransferase showed a dose-related increase in males, which was statistically significant at 100 and 500 mg/kg bw/day (maximum of 242% control). Significant increases were also seen in alanine aminotransferase in females, and in aspartate

aminotransferase of both sexes, but without a clear dose-response relationship. A dose-related increase in blood cholesterol was also seen in male rats, which was statistically significant at 100 and 500 mg/kg bw/day (158% and 205% control, respectively). A smaller significant increase was seen in high dose females only (141% control). Other minor changes were reported, which were generally not dose-related and all values were within the historical control range.
Urinalysis revealed no treatment-related effects except for orange coloration of the urine in most animals of the high dose groups.
At autopsy, there seemed to be a dose-related liver enlargement with an accentuated lobular pattern in both sexes. The increase in absolute liver weights was statistically significant in both sexes at 100 and 500 mg/kg bw/day (male: 113-142% of control; female: 112-132% of control). In addition, the relative liver weight of the 20 mg/kg bw/day males was significantly elevated (110% of control). Significant, but not dose related, increases in relative kidney weight were seen in males at all doses and in females only at the high dose. Spleen and ovarian weights were increased in high dose females. Discoloration of the hair, tail and stomach mucosae were observed in animals treated at 100 and 500 mg/kg bw/day. Other findings were common observations in rats and not dose-related.
Histopathological examination revealed centrilobular or diffuse hepatic cell hypertrophy with centrilobular vacuolisation and multifocal hepatic cell degeneration in most animals at 100 and 500 mg/kg bw/day. Centrilobular cytoplasmic vacuolation was also observed in 2/10 males treated at 20 mg/kg bw/day. Tubular dilatation, tubular epithelial cell vacuolisation and/or degeneration and necrosis were reported in the kidneys of rats of both sexes treated at 500 mg/kg bw/day, and of one female treated at 100 mg/kg bw/day. A dilated kidney pelvis was found in 1 male at 20 mg/kg bw/day.
The study failed to define a NOAEL.
The data are clearly consistent with a dose-related effect on the liver. These effects were seen in some animals at the low dose of 20 mg/kg bw/day. The OECD guideline specifies that histopathology should be conducted on animals of other dose groups if considered necessary to further investigate changes observed in the high dose group.

Ref. : 5.1

Histopathological re-examination of all livers and some kidneys at all doses was performed. In the liver, centrilobular cell hypertrophy was seen in 10/10 male and 4/10 female at the high dose (500 mg/kg bw/day) and in 7/10 male and 10/10 female at the intermediate dose (100 mg/kg bw/day). Centrilobular cell degeneration or necrosis was seen in 9/10 male and 9/10 female at the high and intermediate dose. Diffuse cell hypertrophy was seen in 6/10 females at the high dose. Centrilobular hepatocellular vacuolation was seen in 10/10 male and 9/10 female at the high and in all at the intermediate dose. At 20 mg/kg bw/day, mild to moderate centrilobular hepatocellular vacuolation was seen in 3/10 males. Mild focal coagulative hepatocellular necrosis was seen in a further 3/10 males. One of these had green pigmented macrophages.

Kidneys of all control, all high dose and those from the intermediate (2 female) and low dose (1 male) group that showed macroscopic abnormalities were re-examined. Multifocal tubular epithelial vacuolisation was seen in 3/10 males and 7/10 females at the 500 mg/kg bw/day, 1 female at the 100 mg/kg bw/day. Multifocal tubular epithelial degeneration was seen in 3/10 males and 5/10 females at the 500 mg/kg bw/day, 1 female at the 100 mg/kg bw/day. The study author considered these changes could be treatment related.

Ref. : 15.1

Low dose rat study

Guideline	:	OECD 407 (1981)
Species/strain	:	Sprague Dawley Crl: CD (SD) BR
Group size	:	10 males + 10 females
Test substance	:	IMEXINE FP suspended and homogenised in 0.5% methylcellulose hydrogel
Batch no	:	9060122
Purity	:	> 99 %
Dose levels	:	0, 4, 9 and 20 mg/kg bw/day, 7 days/week by gavage
Exposure period	:	29-30 days
GLP	:	in compliance

Groups of 10 male and 10 female rats were dosed with the test substance by gavage at 0, 4, 9 and 20 mg/kg bw/day, 7 days/week for 29 or 30 days, depending on autopsy date. During the study, clinical signs and mortality were monitored daily and bodyweight and food consumption weekly. During week 4, blood was sampled from the orbital sinus for haematology and blood biochemistry; urine was collected overnight for urinalysis. At the end of the treatment periods, a full autopsy was conducted, recording weights, macroscopic and microscopic examination of major organs (microscopic examination was only conducted for tissues of mid and low dose if macroscopic findings were reported). Ophthalmoscopy was conducted before the start of the study and at the end of the treatment period on all animals.

Results
There were no mortalities or clinical signs of toxicity. Bodyweight gain and food consumption was comparable for all groups. Ophthalmologic examinations revealed no treatment-related signs, minor eye conditions were noted on study day –1, but were resolved at the end of the study period.
Minor changes were reported in some haematological and biochemical parameters but all individual values were within the range of historical controls and not dose-related, and therefore the study author concluded that the changes were not of toxicological significance.
Absolute and relative liver weights showed dose-related increases in both sexes. Dosing at 20 mg/kg bw/day, there were statistically significant increases in both absolute liver weights (male, 119%; female, 118%) and relative liver weights of both sexes (male,120%; female, 117% of control). At 9 mg/kg bw/day, there were lower increases (10%) that were significant for the relative liver weight of males and the absolute liver weight of females.
Histopathological examination revealed hepatocellular vacuolation 1/10 males and 3/10 females of the control groups and 3/10 males and 5/10 females treated at 20 mg/kg bw/day. The study author considered this apparent increase to be of no toxicological importance because it is a common phenomenon in untreated rats. It should be noted that for the low and mid-dose groups, liver sections were examined for one male and no female animals.

There were more incidents of chronic interstitial pneumonia in the high dose animals (3/10 males and 7/10 females) compare with controls (1/10 males and 1/10 females). This observation was considered to be unrelated to treatment by the study authors because it occurs spontaneously in 70% of animals. No low or mid-dose lung sections were examined.
The study author concluded that the high dose of 20 mg/kg bw/day could be defined as the NOAEL.

Ref. : 5.2

Histopathological re-examination of liver at all doses was performed. Mild to moderate centrilobular hepatocellular vacuolation was seen in 5/10 males and 2/10 females treated at 20 mg/kg bw/day, with none evident in the control or lower dose groups. The study author considered this apparent increase to be of no toxicological importance because it is a common phenomenon in untreated rats. All other phenomena were considered to be with the normal range and thus, of no toxicological relevant. No additional assessment of the kidney histopathology was done any dose level. The study author concluded that the 20 mg/kg bw/day dose was the NOAEL.

Ref. : 15.2

The conclusions of the study authors from the two studies and the re-evaluation of the histopathology are conflicting. The two studies showed a remarkably consistent hepatic response at 20 mg/kg bw/day, which was considered to be treatment-related in the high dose study, and irrelevant in the second study. The OECD guideline specifies that histopathology should be conducted on animals of other dose groups if considered necessary to further investigate changes observed in the high dose group. In this instance, the kidney and lung from the low and mid-dose group (4 and 9 mg/kg bw/day from the low dose study) should have been provided to support the study author's conclusion that the effects at the lower doses are not toxicologically relevant. Since this was not done, the SCCNFP concluded that the NOAEL should be 9 mg/kg bw/day.

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

No data

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)

Guideline	:	OECD 404 (1981)
Species/strain	:	New Zealand albino rabbit
Group size	:	3 males at each time point
Test substance	:	IMEXINE FP suspended at 3% in 1,2-propanediol
Batch no	:	9060122
Purity	:	> 99 %
Dose	:	0.5 ml
GLP	:	in compliance

The substance was suspended in 1,2-propanediol at a concentration of 3%. 0.5 ml was applied to a 6 cm^2 area of intact skin of 3 male rabbits. Semi-occlusive patches were applied and left in place for 4 hours and then remaining test substance was rinsed off. The skin was examined for erythema, eschar formation and oedema at 1, 24, 48 and 72 hours and then daily after removal of the patches.

Results
A very slight erythema was observed in 2 animals after one hour, which persisted to 24 or 48 hours. The third animal exhibited a well-defined erythema at 1 hour, which was remained as slight erythema until day 6, accompanied by dryness on days 4-6. According to the classification system defined in 83/467/EEC, the substance was classified as non-irritating to rabbit skin at a concentration of 3% in 1,2-propanediol.
The substance should be considered slightly irritating to rabbit skin.

Ref. : 3

2.4.2. Irritation (mucous membranes)

Guideline	:	OECD 405 (1987)
Species/strain	:	New Zealand albino rabbit
Group size	:	3 males
Test substance	:	IMEXINE FP suspended at 3% in 1,2-propanediol
Batch no	:	9060122
Purity	:	> 99 %
Dose	:	0.1 ml
GLP	:	in compliance

The test substance was suspended in 1,2-propanediol at a concentration of 3%. 0.1 ml was applied once to the left eye of 3 male rabbits, without rinsing. The right eye served as control and was untreated. Ocular reactions were recorded at 1, 24, 48 and 72 hours after instillation.

Results
Slight conjunctival reactions were observed after one hour in all 3 rabbits and the reaction persisted to 24 hours in 2 animal. There were no iridial or corneal reactions.

Ref. : 2

2.5. Sensitisation

Magnusson and Kligman study

Guideline	:	OECD 406 (1981)
Species/strain	:	Dunkin Hartley guinea pig
Group size	:	10 males + 10 females in test group, 5 male + 5 female in control group
Test substance	:	IMEXINE FP suspended in 1,2-propanediol
Batch no	:	9060122
Purity	:	> 99 %
Concentrations	:	intradermal induction : 0.1 ml Freund's complete adjuvant (FCA)
		0.1 ml 1% test substance
		0.1 ml % test substance/FCA (1:1)
		induction of irritation : 0.5 ml of 10% sodium lauryl sulphate in vaseline
		topical induction : 0.5 ml 3% test substance for 48 hours, occluded
		challenge : 0.5 ml 3% test substance for 24 hours, occluded
GLP	:	in compliance

Induction commenced with three intradermal injections, of FCA, test substance (1.0%), and a mixture of these two. On day 6, 0.5 ml of 10% lauryl sulphate was applied to the injection site to induce a local irritation. On day 7, the induction process was completed with a single topical application of 0.5ml of the test substance (3%) under occlusive patch for 48 hours. Two weeks after induction, the animals were challenged by a single 0.5 ml topical application of the test substance (3%) under occlusive patch on the flank for 24 hours. Appropriate controls were treated with vehicle at all stages. The skin was examined 24 and 48 hours after removal of the challenge patches.

Results
Irritation was observed at the intradermal injection site at the end of the induction period. The 24 hour evaluation was obscured by yellow staining of the skin. There was no interference at 48 hours, at which time there was no evidence of cutaneous reaction and it was therefore concluded that the substance did not exhibit sensitising potential under the conditions of the experiment.

Ref. : 4

2.6. Teratogenicity

Guideline	:	OECD 414 (1981)
Species/strain	:	Sprague-Dawley rat, Crl: CD (SD) BR
Group size	:	25 females (mated)
Test substance	:	IMEXINE FP suspended and homogenised in 0.5% methylcellulose hydrogel
Batch no	:	9060122
Purity	:	> 99 %
Dose levels	:	0, 10, 30 and 90 mg/kg bw/day
Treatment period	:	Days 6 to 15 of pregnancy, inclusive
GLP	:	in compliance

The test substance was given by gavage at 0, 10, 30 and 90 mg/kg bw/day on days 6 to 15 after mating to 25 female rats per dose. The dams were observed daily for clinical signs and mortality. Bodyweight and food consumption were recorded on pregnancy days 0, 6, 11, 16 and 20. They were sacrificed on day 20 of pregnancy, and examined for number of corpora lutea, number and distribution of live and dead foetuses, of early or late resorptions and of implantation sites, and for macroscopic observations. The bodyweight, sex and macroscopic external observations of all foetuses were recorded. The foetuses were checked for skeletal and visceral abnormalities (half for each endpoint). The concentrations, homogeneity and stability of the dosing formulations were determined, but the results were not given.

Results
There were no premature deaths and no treatment-related clinical signs except for orange coloration of the urine from day 7 to 16 of pregnancy in all treated groups. The high dose group animals exhibited reduced weight gain compared to controls from day 6 to 12. This was not statistically significant, but actual body weight was significantly lower on day 12 (95% of control). Food consumption was significantly decreased in the 90 mg/kg bw/day dose group from day 6 to 15 and in the 30 mg/kg bw/day dose group from day 12-15.
There were no treatment-related observations in the dams at autopsy. The mean numbers of corpora lutea, implantation sites, post-implantation loss, live foetuses, sex distribution and the mean foetal bodyweights were not significantly different for control and treated groups. There were no dose-related increases in foetal abnormalities or malformations. Some individual significant differences were observed but these were within the historical control range and considered not to be dose-related. The incidence of anomalies and malformations were lower in the high dose group than in the control group, but this was not significant.
The test substance elicited a slight maternal effect at 90 mg/kg bw/day, but not embryo-toxic or teratogenic.

Ref. : 11

2.7. Toxicokinetics (incl. Percutaneous Absorption)

2.7.1 Percutaneous Absorption *in vitro*

Study 1

Guideline	:	/
Tissue	:	Human abdominal epidermis, heat-separated
Method	:	Franz diffusion cell (static)
Test substance	:	6-nitro-o-toluidine, 0.90% in formulation
Batch no	:	9060122 21
Purity	:	> 99 %
Dose levels	:	circa 40 mg formulation in the presence/absence of 10 mg hair
Replicate cells	:	8 cells with hair and without hair
GLP	:	not in compliance

The skin penetration of 6-nitro-o-toluidine was evaluated in a static Franz diffusion cell system. Human epidermis was prepared by heat-separation from previously frozen abdominal skin. The test substance was prepared at a concentration of 0.9% in a formulation. Approximately 40 mg of the mixture was applied to 2cm^2 of epidermal membrane with and without addition of 10 mg

finely chopped bleached hair for 30 minutes and then excess washed off with 2% sodium lauryl sulphate solution and dried. Four hours later the levels of substance were measured in the receptor fluid (physiological saline) using HPLC. Integrity of the epidermal membrane was checked by microscopy before the study, and by means of addition of Chinese ink at the end of the study. Any cells showing penetration of the ink were eliminated from the analysis.

Results
The quantity of test substance penetrating through the epidermis to the receptor fluid corresponded to 1.77% of applied dose in the presence of hair and 3.15% in the absence of hair. This study did not include determination of recovery of the test substance and insufficient time was allowed for permeation to the receptor fluid. Physiological saline was used as the receptor fluid. No information has been provided on the water solubility of this substance, and based upon the choice of vehicles used in the toxicity studies, it must be assumed that it is poor. It is likely therefore that the receptor fluid is not appropriate and that a higher rate of penetration may be achieved under different conditions.
The study is considered inadequate.

Ref. : 13

Study 2

Guideline	:	/
Tissue	:	Human abdominal dermatomed skin
Method	:	Franz diffusion cell (static)
Test substance	:	IMEXINE FP at the concentration of 0.69 % w/w in commercial formulation batch number 443963
Batch no	:	00290T0001
Purity	:	/
Dose levels	:	20 mg/cm²
Replicate cells	:	4 skin donors, 2 cells/donor, 7 cells interpreted (8 cells performed, one discarded because aberrant value after statistical analysis with the Dixon's test
Analytical method	:	HPLC (validated) with UV-Visible detection Limit of detection : not indicated limit of quantitation : 15 ng/ml
Stability	:	stability of IMEXINE FP in the formulation was evaluated after 1 month at 45°C plus 1 month at room temperature : there was a loss of 6 %
GLP	:	in compliance

The skin penetration of 6-nitro-o-toluidine was evaluated in a static Franz diffusion cell system. Human abdominal skin previously frozen was dermatomed to a constant thickness. The integrity of the skin was evaluated by the measurement of the TEWL. The solubility of 6-nitro-o-toluidine in the receptor fluid (physiological saline) was checked in the range of the concentration used. The test substance was prepared at a concentration of 0.69 % in a formulation. Approximately 20.43 ± 0.37 mg/cm² of the formulation i.e. 139.54 ± 3.88 µg/cm² (exactly measured by weight) were applied to 2 cm² for 30 minutes. The excess from the skin surface was rinsed first with water, followed by washing with 2 % sodium lauryl sulphate aqueous solution, again rinsed with water and finally dried with a cotton swab. 24 hours after the application the substance was

measured using HPLC in the receptor fluid, in the horny layer collected by tape stripping (10 to 15 strips), in the epidermis/ dermis and in the remaining skin outside the application area. After assay of 6-nitro-o-toluidine in the washing material (skin excess) the mass balance of the study was calculated.
Results
The quantity of test substance penetrating through the skin to the receptor fluid corresponded to 2.62 ± 1.42 % of the applied dose (3.65 ± 2.00 µg/cm²). The amount recovered in the horny layer was 0.11 ± 0.19 % of the applied dose (0.16 ± 0.26 µg/cm²). The epidermis and the dermis content was 0.11 ± 0.03 % of the applied dose (0.16 ± 0.03 µg/cm²). The recovery of the test substance is 101.29 ± 8.55 % of the applied dose. No storage of the compound in the stratum corneum and the epidermis/dermis occurred.
The absorbed amounts of 6-nitro-o-toluidine (epidermis + dermis + receptor fluid) represents 2.73 ± 1.44 % of the applied dose (3.81 ± 2.02 µg/cm²) at the end of 24 hours of diffusion after contact with the skin of 30 minutes.

Ref: 14

2.8. Mutagenicity/Genotoxicity

2.8.1 Mutagenicity/Genotoxicity *in vitro*

Bacterial reverse mutation test

Guideline	:	OECD 471 (1983)
Species/strain	:	*S. typhimurium*, TA98, TA100, TA1535, TA1537; *E. coli*, WP2uvrA
Replicates	:	Triplicate plates, 2 independent tests
Test substance	:	IMEXINE FP in DMSO solution
Batch no	:	9060122
Purity	:	> 99 %
Concentrations	:	156 - 2500 µg/plate with and without metabolic activation
GLP	:	in compliance

6-Nitro-o-toluidine has been investigated for gene mutation in *S. typhimurium* and *E. coli* using a plate incorporation protocol, and a preincubation step in one study with metabolic activation. Liver S9 fraction from Aroclor 1254-induced rats was used as the exogenous metabolic activation system. A preliminary study showed toxicity at 2500 and 5000 µg/plate and therefore the concentration range was initially based on a maximum of 2500 µg/plate. Negative and positive controls were in accordance with the OECD guideline.

Results
The test substance increased numbers of revertant colonies in TA98 in the presence and absence of S9, and in TA100 in the absence of S9. The positive control agents gave the expected results. The compound is considered mutagenic in this test system.

Ref. : 6

In vitro Mammalian Cell Gene Mutation test

Guideline : OECD 476 (1984)

Species/strain	:	Mouse lymphoma L5178Y $TK^{+/-}$ cells
Replicates	:	2 independent tests
Test substance	:	IMEXINE FP in DMSO solution
Batch no	:	9060122
Purity	:	> 99 %
Concentr. scored	:	3 – 600 μg/ml without metabolic activation, 3 – 300 μg/ml with activation; 3-300 μg/ml without S9; 10-400 μg/ml with S9
GLP	:	in compliance

6-Nitro-o-toluidine has been investigated for induction of cell mutations at the TK locus in mouse lymphoma L5178Y $TK^{+/-}$ cells.
Liver S9 fraction from Wistar rats induced with Aroclor 1254 was used as the exogenous metabolic activation system. Test concentrations were selected on the basis of a preliminary toxicity study which established that toxicity started to occur at 300 μg/ml, 4 hours of exposure. Negative and positive controls were in accordance with the OECD guideline.

Results
There were no dose-related or reproducible increases in mutant colonies, with or without metabolic activation. The positive control agents gave the expected results. The compound is considered non mutagenic in this test system.

Ref. : 7

In vitro Mammalian Chromosome Aberration test

Guideline	:	OECD 473
Species/strain	:	Chinese Hamster Ovary Cells
Replicates	:	Duplicate cultures, two independent tests
Test substance	:	1-amino-2-methyl-6-nitrobenzene in DMSO solution
Batch no	:	906122
Purity	:	/
Concentrations	:	250, 500 and 2500 μg/ml in expt 1 and with activation in expt 2 12.5, 62.5 and 125 μg/ml without metabolic activation in expt 2
GLP	:	in compliance

6-Nitro-o-toluidine has been investigated for induction of chromosomal aberrations in CHO cells. Liver S9 fraction from rats induced with β-naphthoflavone/phenobarbitone was used as the exogenous metabolic activation system. The test concentrations were established from a preliminary toxicity study, which resulted in a mitotic index in excess of 50% of control at 2500 μg/ml and compound precipitation at 5000 μg/ml. Exposure was for 24 hours without S9 and 3 hours with S9 and cells were harvested 24 and 48 hours after the start of exposure. Negative and positive controls were in accordance with the OECD guideline.

Results
In the first experiment there were fewer scorable cells than anticipated from the preliminary toxicity study. Statistically significant increases in aberrations were seen with treatment at 500 and 2500 μg/ml in the absence of S9 at the second harvest time. In the second experiment, lower concentrations were used in the absence of S9 and there was no increase in aberrations. There were significant increases in aberration frequency at both harvest times in the presence of

metabolic activation in the second experiment, with higher incidence at the second harvest. The test substance showed clastogenic potential, associated with lower numbers of scorable cells and possibly related to an effect on the cell cycle. The positive control agent gave the expected result. The compound is considered clastogenic in this test system.

Ref. : 8

2.8.2 Mutagenicity/Genotoxicity *in vivo*

Mammalian Erythrocyte Micronucleus test

Guideline	:	OECD 474 (1981)
Species/strain	:	Mouse, Swiss OF1/ICO: OF1 (IOPS Caw) strain
Group size	:	5 male + 5 female (supplementary 3 male + 3 female at high dose)
Test substance	:	IMEXINE FP suspended in 0.5% aqueous carboxymethylcellulose
Batch no	:	9060122
Purity	:	> 99 %
Dose levels	:	0, 400, 800 and 1600 mg/kg bw, two doses at 24 hour interval
Sacrifice times	:	24 hours after second dose
GLP	:	in compliance

6-Nitro-o-toluidine has been investigated for induction of micronuclei in the bone marrow cells of mice. Dose levels were determined by a preliminary toxicity study in which 1/6 mice died at 2000 mg/kg bw/day and clinical signs of toxicity were apparent in 3/6 mice at 1500 mg/kg bw/day. The substance was administered twice at 24 hour intervals and the bone marrow harvested 24 hours after the second dose. Negative and positive controls were in accordance with the OECD guideline.

Results:
There was no evidence of increased incidence of micronucleated polychromatic erythrocytes in any of the test groups. The ratio of polychromatic to normochromatic erythrocytes was significantly decreased at the top dose, demonstrating that the test substance effectively reached the bone marrow.
The compound is considered non-clastogenic in somatic cells *in vivo*.

Ref. : 9

Unscheduled DNA Synthesis (UDS) with Mammalian Liver Cells

Guideline	:	draft OECD guideline of 1991 (475)
Species/strain	:	Wistar rat, HanIbm: WIST (SPF) strain
Group size	:	3 males
Test substance	:	IMEXINE FP in DMSO solution
Batch no	:	9060122
Purity	:	> 99 %
Dose levels	:	0, 100 and 1000 mg/kg bw
Sacrifice times	:	16 hours: all dose groups; 2h: high dose group
GLP	:	in compliance

6-Nitro-o-toluidine has been investigated for induction of unscheduled DNA synthesis (UDS) in rat hepatocytes *in vitro* following *in vivo* dosing. A preliminary toxicity study resulted in death of one of two animals at 2000 mg/kg bw, and persistent clinical signs of toxicity at 1500 mg/kg bw and transient signs at 1000 mg/kg bw, which was subsequently selected as the upper dose for the UDS study. Negative and positive controls were in accordance with the OECD guideline. Animals were sacrificed after 16 hours, and for an additional high dose group after 2 hours. Four animals were dosed per group, and three of them used for isolation of hepatocytes, which were then treated with ^{3}H-thymidine *in vitro*. Incorporation of radio-label was assessed using autoradiography.

Results
There were no differences in the viability of hepatocytes isolated from rats of different dose groups. The results met all the pre-defined criteria for a negative response and therefore the test substance was not found to induce UDS. The positive control agent gave the expected results. The compound is considered non-genotoxic in somatic cells *in vivo*.

Ref. : 10

2.9. Carcinogenicity

No studies are available on 6-Nitro-o-toluidine.

2,3-Dinitrotoluene is formed if the amino-group is oxidised. This substance is classified as a carcinogen category 2 and a mutagen category 3 by EU.

The metabolism of dinitrotoluenes begins in the liver, where they are oxidised by cytochrome P450 and conjugated with glucuronic acid to form the major metabolite dinitrobenzyl alcohol glucuronide and excreted in bile or urine.

Ref. : d, e

The glucuronide excreted in bile undergoes biotransformation by intestinal microflora, where the conjugate is hydrolyzed and subsequently reduced by nitroreductase to the corresponding aminonitrobenzyl alcohol. 6-Nitro-o-toluidine may give rise to the same aminonitrobenzyl alcohol as 2,3-dinitrotoluene.

Ref. : a, b, f

The aminonitrobenzyl alcohol is reabsorbed and transported back to the liver by enterohepatic circulation.

Ref. : e

The amine group is subsequently N-hydroxylated by cytochrome P450 and conjugated with sulfate.

Ref. : c

The sulfate conjugate is unstable and can be decomposed to form a carbonium or nitrenium ion that can be bound to macromolecules; this ostensibly leads to mutations and the formation of tumours. In agreement with this it is found that 5-nitro-o-toluidine is carcinogenic (Classified as carcinogen category 3 by EU and category 2 by the MAK-Committee in Germany).

It should also be noted that if the nitro-group of 6-nitro-o-toluidine is reduced 2,3-diaminotoluene is formed. The EU has classified diaminotoluenes as carcinogen category 2.

2.10. Special investigations

6-Nitro-o-toluidine has been tested for eye irritation potential using the hen's egg chorio-allantoic membrane (HET-CAM) test. The substance was either applied neat (0.1g), and washed off after 20 seconds, or as 0.3 ml of a 10% solution in paraffin oil. The membranes were observed for 5 minutes for potential reactions. There was no response and the authors concluded that the substance was non-irritant by the ocular route. The study did not include positive control agents and the report did not include historical control data on response to positive control agents.
The HET-CAM assay has been extensively used and is showing promise as a potential alternative assay for eye irritation. However, it has not yet been validated, and it is essential that positive control agents should be included in order to demonstrate the sensitivity of the assay. This study does not extend the information on eye irritation provided by the rabbit study reported above in section 4.2 and is not helpful in the safety evaluation of the substance.

Ref. : 12

2.11. Safety evaluation

NOT APPLICABLE

2.12. Conclusions

The degradation products have not been characterised, despite 6 % loss of the test material noted in the formulation used in the percutaneous absorption study over time.

Two 28-day repeated dose oral studies were conducted. The first of these provide clear evidence of dose-related hepatotoxicity and concluded that the lowest dose tested, 20 mg/kg bw/day was a LOAEL. The study was therefore repeated using a lower dose range, with 20 mg/kg bw/day as the highest dose. A similar incidence and severity of liver effect were seen at 20 mg/kg bw/day. Despite this, the study authors concluded the NOAEL was 20 mg/kg bw/day. However the information provided does not justify this conclusion. In the absence of sub-chronic studies, a NOAEL of 9 mg/kg bw/day should be assumed.
When administered during organogenesis, the substance slightly affected maternal food consumption and weight gain, with a NOAEL of 30 mg/kg bw/day. There was no evidence of foetotoxicity or teratogenicity.

6-Nitro-o-toluidine was slightly irritating to rabbit skin and eye when applied at a concentration of 3% in 1,2-propanediol and has not shown evidence of sensitisation.
In vitro percutaneous absorption of a 0.69% solution of 6-nitro-o-toludine in a hair dye formulation was 3.81 ± 2.02 $\mu g/cm^2/24h$.

The substance induced gene mutations in bacteria and clastogenicity in mammalian cells *in vitro*. Two *in vivo* genotoxicity studies, using complementary species and endpoints, indicated that the *in vitro* mutagenic potential is not expressed *in vivo*.
The compound is considered a non-clastogenic and non-DNA damaging agent in somatic cells *in vivo*.

In the absence of data on metabolic fate *in vivo*, 6-nitro-o-toluidine may be carcinogenic.

2.13. References

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SCCNFP

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3. Opinion of the SCCNFP

The SCCNFP is of the opinion that the information submitted is inadequate to allow a risk assessment to be carried out.

Consequently, and before any further consideration, the following is required :

* data on the characterisation of the degradation products of 6-nitro-o-toluidine.

* the potential carcinogenicity of 6-nitro-o-toluidine and its metabolites should be clarified.

* 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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