EUROPEAN COMMISSION

Health & Consumer Protection
Directorate-General

Scientific Committee on Consumer Products

SCCP

OPINION ON

2-Nitro-5-glyceryl methylaniline

COLIPA n° B60

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

The SCCP adopted this opinion at its 19th plenary of 21 January 2009

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

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

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

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

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Opinion to be cited as: SCCP (Scientific Committee on Consumer Products), Opinion on 2-nitro-5-glyceryl methylaniline, 21 January 2009

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1. BACKGROUND

Submission I and II for 2-Nitro-5-glyceryl methylaniline were submitted by COLIPA[1] in July 1993 and in November 2001 respectively.

The Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers (SCCNFP) adopted at its 24th plenary meeting of 24-25 June 2003 the opinion (SCCNFP/0688/03, final) with the conclusion:

"The SCCNFP is of the opinion that the information submitted is inadequate to assess the safe use of the substance. Before any further consideration, the following information is required:

* *complete data on physico-chemical and chemical characterisation of the test material; nitrosamine content in various batches of 2-nitro-5-glyceryl methylamine and in hair dye formulations containing this chemical;*
* *percutaneous absorption study in accordance with the Notes of Guidance;*
* *data on genotoxicity/mutagenicity following the relevant SCCNFP opinions and in accordance with the Notes of Guidance."*

According to the current submission III, submitted by COLIPA in July 2005, the substance is used in semi permanent hair colouring products at a maximum concentration of 1.0%.

Submission III presents updated scientific data on the above mentioned substance in line with the second step of the strategy for the evaluation of hair dyes (http://europa.eu.int/comm/enterprise/cosmetics/doc/hairdyestrategyinternet.pdf) within the framework of the Cosmetics Directive 76/768/EEC.

2. TERMS OF REFERENCE

1. *Does the Scientific Committee on Consumer Products (SCCP) consider 2-Nitro-5-glyceryl methylaniline safe for use as a non-oxidative hair dye with an on-head concentration of maximum 1.0 % taking into account the scientific data provided?*

2. *Does the SCCP recommend any restrictions with regard to the use of 2-Nitro-5-glyceryl methylaniline in any hair dye formulations?*

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association

3. OPINION

3.1. Chemical and Physical Specifications

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

2-Nitro-5-glyceryl methylaniline (INCI name)

3.1.1.2. Chemical names

1-Methylamino-2-nitro-5-(2,3-dihydroxy-propyloxy)-benzene
3-[3-(methylamino)-4-nitrophenoxy]-propane-1,2-diol

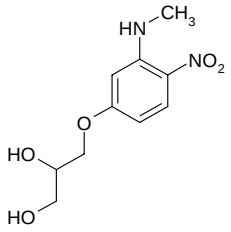
3.1.1.3. Trade names and abbreviations

Imexine FT

3.1.1.4. CAS / EINECS number

CAS: 80062-31-3
EINECS: 279-383-3

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_{10}H_{14}N_2O_5$

3.1.2. Physical form

Yellow powder with green highlights

3.1.3. Molecular weight

Molecular weight: 242 g/mol

3.1.4. Purity, composition and substance codes

The analytical study of 2-Nitro-5-glyceryl methylaniline was carried out on five batches:

- Batch Op. 8 refers to an analytical certificate (April 1990)
- Batch Op. T107 refers to analytical certificate (October 1993)

- Batch Op. 77 refers to an analytical certificate (September 1994)
- Batch 0504494 refers to an analytical certificate (January 2001)
- Batch 0122117, results of an analytical study provided (no date)

Description/Batch No.	OP.8	Op.77	Op.T107	0504494	0122117
Identification		NMR, IR, MS, Vis-spectrometry			NMR, IR, MS, Vis-spectrometry, Elemental analysis
Content					
UV-Vis spectrometry, content at 413 nm (w/w)	> 99	98	98.3	98	98.4
HPLC (% peak area)	> 99.5	> 99.5			> 99.5
Water content, %(w/w)		0.34	0.06		0.1
Ash content, %(w/w)					< 0.1
Impurities, % (w/w)					
A*				< 0.020	< 0.010 ND
B*				< 0.020	< 0.010 ND
C		0.0075	0.004		< 0.010 ND
D		0.095	0.12	< 0.25	< 0.010 D
E				< 0.4	< 0.010 D
F*		< 0.01			
G*		< 0.01			
Residual solvent ppm					
Isopropanol		100			
Methanol		< 50			
Toluene		330			
Solketal**					500

ND: Not detected
D: Detected
* The synthetic pathway leading to batches 0504494 and 0122117 was different from the synthetic pathway leading to batches Op. 8, Op. T107 and Op. 77. Hence, impurities A and B were checked only in batches 0504494 and 0122117 and impurities F and G in batch Op. 77, respectively.
Possible impurities which may originate from reagents and intermediate reaction products were checked:
A: 2,4-difluoro-1-nitrobenzene
B: 5-fluoro-2-nitrophenyl)-methylamine
C: 3-methylamino-4-nitrophenol or (5-hydroxy-2-nitrophenyl) methylamine
D: 1-(3-methylamino-4-nitrophenyl)-2,3-isopropylidenglycerol or 5-[2,2-dimethyl-(1,3)dioxolan-4-ylmethoxy]-2-nitrophenylmethylamine
E: 2,4-dimethylaminonitrobenzene
F: 2,4-dichloro-1-nitrobenzene
G: 5-chloro-2-nitrophenyl)-methylamine
** Solketal: (2,2-dimethyl-1,3-dioxolan-4-yl)methanol

3.1.5. Impurities / accompanying contaminants

See 3.1.4

Heavy Metals in batch n° 0122117:
- As, Sb, Hg: each < 5 mg/k
- Cd: < 10 mg/kg
- Pb: < 20 mg/kg

Ash content: < 0.1 g/100g

3.1.6. Solubility

Water:	1.43 g/l	at 20°C	EEC method A6
Ethanol:	< 1 g/100ml	at 22°C after 24 h	
DMSO:	> 20 g/100 ml	at 22°C after 24 h	

3.1.7. Partition coefficient (Log P_{ow})

Log Po/w:	1.14	at 25°C and pH 6.19	EEC method A8

3.1.8. Additional physical and chemical specifications

Melting point:	95-97 °C (thermo-microscopic method) 105 °C (differential calorimetry)
Boiling point:	
Flash point:	
Vapour pressure:	
Density:	
Viscosity:	
pKa:	
Refractive index:	
pH:	
UV_Vis spectrum (200-800 nm):	absorbance at 233.9 (λmax), 253.9, 312.2 and 412.6 nm

3.1.9. Homogeneity and Stability

The homogeneity testing of 2-Nitro-5-glyceryl methylaniline (batch 0122117) was performed at 1 and 100 mg/mL in 0.5% aqueous methylcellulose (MC) on the day of preparation. The stability testing of 2-Nitro-5-glyceryl methylaniline (batch 0122117) in dosage forms was performed at 1 and 100 mg/mL in 0.5% MC, at 0.1 and 250 mg/mL in DMSO and at 1 and 250 mg/mL in DMF over a 4-hour period at room temperature, protected from light and under inert gas atmosphere. The deviation from nominal concentrations in all cases was maximum 9%.

General Comments to physico-chemical characterisation

- 2-Nitro-5-glyceryl methylaniline is a secondary amine, and thus, it is prone to nitrosation. Nitrosamine content in the test material is not reported.
- The stability of 2-Nitro-5-glyceryl methylaniline in the marketed products is not reported

3.2. Function and uses

2-Nitro-5-glyceryl methylaniline is used in semi permanent hair colouring products at a maximum concentration of 1%. The formulation is applied as such without any further dilution.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Guideline:	OECD 401
Species/strain:	Sprague – Dawley, fasted
Group size:	10 per sex
Test substance:	IMEXINE FT
Batch:	Op. 8
Purity:	99.9%
Dose:	1000 mg/kg and 2000 mg/kg
Route:	Oral
Exposure:	single administration and 14-days observation
GLP:	in compliance
Study period:	April 1992

Ten Sprague-Dawley rats per sex were exposed to 2-nitro-5-glyceryl methylaniline (B060) at the dose of 1000 mg/kg and 2000 mg/kg in 1% aqueous methylcellulose. Animals were observed twice daily for mortality/morbidity and daily for clinical signs over a period of 14 days. Body weights were recorded on day 1 prior to treatment, and on days 5, 8 and 15 thereafter. All study animals were subjected to a macroscopic examination.

Results
At 2000 mg/kg, 4/5 males and 5/5 females died 24 to 48 hours after treatment. Sedation and dyspnoea were observed 15 minutes after exposure at both dose levels. Ptosis was also observed in a few animals exposed to 2000 mg/kg. No mortality was observed at 1000 mg/kg/day, where clinical signs included sedation, dyspnoea, lateral recumbency and orange colouration of extremities (reversible from day 3 onwards). No effect on body weight gain was observed at either dose level. At necropsy, black areas were noted on the stomach in three animals treated at 2000 mg/kg; orange discolouration of all tissues and organs was observed in animals found dead.

Conclusion
The maximal non-lethal dose of B060 was higher than 1000 mg/kg and lower than 2000 mg/kg after a single oral administration in fasted rats.

Ref.: 1

3.3.1.2. Acute dermal toxicity

No data submitted

3.3.1.3. Acute inhalation toxicity

No data submitted

3.3.2 Irritation and corrosivity

3.3.2.1. Skin irritation

Taken from SCCNFP/0688/03

Guideline: OECD n° 404

Species:	rabbit (New Zealand white)
Group size:	3 males
Substance:	IMEXINE FT at 1% in 1,2-propanediol
Batch:	op 8
Purity:	99.9%
Dose:	0.5 ml of a 1% suspension
GLP:	in compliance
Study period:	March 1992

0.5 ml of a 1% suspension of the test substance in 1,2-propanediol was applied to the right clipped dorsal flank of 3 male rabbits. The left flank did not receive any substance and served as a control. The test substance was kept in contact with the skin for 4 hours under a semi-occlusive patch. Any reactions were evaluated 60 minutes and 24, 48 and 72 hours following the removal of the semi-occlusive dressing. At termination, histopathological examination of the treated skin was carried out.

Results
No oedema was noted in any of the 3 animals. Reddish coloration of the skin made it impossible to evaluate erythema. Histological examination of samples of treated skin revealed no relevant abnormalities.

Conclusion
The test substance was considered as non-irritant to rabbit skin when applied as a 1% suspension in 1,2-propanediol.

Ref.: 2

3.3.2.2. Mucous membrane irritation

Taken from SCCNFP/0688/03

Guideline:	OECD n° 405
Species:	rabbit (New Zealand white)
Route:	eye
Group size:	3 males
Substance:	IMEXINE FT, 1% suspended in 1,2-propanediol
Batch:	op 8
Purity:	99.9%
Dose:	0.1 ml of a 1% suspension
GLP:	in compliance
Study period:	March 1992

0.1 ml of a 1% suspension of the test substance in 1,2-propanediol was instilled into the left eye of 3 male rabbits, the right eyes served as control. The material was not rinsed out. Eye irritation was scored one hour, 24, 48 and 72 hours following instillation.

Results
One hour after instillation, slight chemosis and redness of the conjunctivae were observed. After 24, 48 and 72 hours, no reactions to the conjunctivae were seen. No irritation of the iris and no corneal opacity were noted.

Conclusion
The test substance was considered as non-irritant to the rabbit eye when applied as a 1% suspension in 1,2-propanediol.

Ref.: 3

Comment

The SCCP concluded that a 1% suspension caused some slight and transient irritation of the rabbit eye.

3.3.3. Skin sensitisation

Local Lymph Node Assay (LLNA)

Guideline:	OECD 429 (2002)
Species:	CBA/J mouse, nulliparous and non-pregnant females
Group:	28 females (5 test groups, 1 positive control group, 1 vehicle control group)
Substance:	2-nitro-5-glyceryl methylaniline
Batch:	0122117
Purity:	98.4%
Concentrations:	0.5, 1, 2.5, 5 and 10% in dimethylformamide (DMF)
Dose:	25 µl
Vehicle:	dimethylformamide (DMF)
Control:	α-hexylcinnamaldehyde (HCA), 25% in DMF
GLP:	in compliance
Study period:	24 August – 6 September 2004

Twenty-eight female CBA/J mice were allocated to seven groups:

- five treated groups of four animals receiving the test item 2-nitro-5-glyceryl methylaniline (B060) at the concentration of 0.5, 1, 2.5, 5 or 10%,
- one negative control group of four animals receiving the vehicle (DMF),
- one positive control group of four animals receiving the reference item, HCA, a moderate sensitizer, at the concentration of 25% in DMF.

During the induction phase, the test item, vehicle or reference item was applied over the ears (25 µL per ear) for 3 consecutive days (days 1, 2 and 3).
On day 6, the mice received an intravenous injection of 250 µl of 0.9% NaCl containing 20 µCi of tritiated methyl thymidine. Approximately five hours later, the mice were sacrificed by cervical dislocation and the auricular lymph nodes were excised. The lymph nodes were pooled for each experimental group. The proliferation of lymphocytes was measured by incorporation of tritiated methyl thymidine. The obtained values were used to calculate stimulation indices (SI).

Results
Systemic clinical signs and mortality: no clinical signs and no mortality related to treatment with the test item were noted.

Proliferation assay: the stimulation index (SI) was below 3 in all test groups (see table). The positive control (HCA at 25% in DMF) caused a SI of 7.47. No EC3 value was calculated, since all stimulation indices were below 3.

The results are presented in the following table:

Concentration (%)	Stimulation index
0.5	1.67
1.0	1.80
2.5	2.04
5.0	1.38
10.0	0.98
25% α-hexylcinnamaldehyde	7.47

Conclusion
Under the conditions of this Local Lymph Node Assay, 2-nitro-5-glyceryl methylaniline did not induce skin sensitisation.

Ref.: 4

Comment
The highest concentration of the test substance (10%) tested is considered too low. 25% was not tested, justified by the authors by reference to skin irritancy (moderate increase in ear thickness) in the preliminary test to that concentration. The restriction is not according to the test guideline which states that exposure shall be maximized whilst avoiding systemic toxicity and excessive local skin irritation.
No conclusion regarding the sensitising potential of 2-nitro-5-glyceryl methylaniline can be drawn.

3.3.4. Dermal / percutaneous absorption

Guideline:	OECD draft 428 (2000)
Tissue:	dermatomed human (female) abdominal skin, 400 µm thickness
Group size:	8 skin preparations from 4 different donors
Diffusion cells:	9 mm flow-through diffusion cell, 0.64 cm² area
Skin integrity:	permeation coefficient for tritiated water (< 2.5 x 10^{-3} cm/hour)
Test substance:	2-nitro-5-glyceryl-methylaniline [^{14}C]- 2-nitro-5-glyceryl-methylaniline, 8.46 MBq/mg, 2064.4 MBq/mmol
Batch:	0122117 SEL/1439 (radio-labelled)
Purity:	98.4% 98.4% (HPLC) (radio-labelled)
Test item:	formulation 1036515, 0.9% (w/w) test substance
Doses:	20 mg/cm²
Receptor fluid:	phosphate-buffered saline containing 0.01% sodium azide (w/v) and 5% bovine serum albumin
Solubility receptor fluid:	650 µg/ml
Stability:	considered sufficient during 'life phase' of study
Method of Analysis:	liquid scintillation counting
GLP:	in compliance
Study period:	24 – 31 January 2005

The *in vitro* percutaneous absorption of 2-nitro-5-glyceryl-methylaniline in a semi-permanent hair dye formulation was determined in human dermatomed skin mounted in flow-through diffusion cells.

The integrity of 20 skin samples was established by determination of the permeation coefficient for tritiated water. From these, 8 skin samples (2 from each of the 4 donors) with a permeation coefficient of < 2.5 x 10-3 cm.h^{-1} were selected for the study.

20 mg/cm^2 of a formulation containing 0.9% 2-nitro-5-glyceryl-methylaniline was applied on the skin surface. After the 30-minute exposure period, the test substance was removed from the application site by washing and using cotton swabs. Twenty-four hours after application, each skin was tape stripped 10 times per skin membrane. The cutaneous distribution of 2-nitro-5-glyceryl-methylaniline was assessed by Liquid Scintillation Counting in the skin wash, stratum corneum (isolated by tape stripping), skin samples and receptor fluid.

Results

Most of the 2-nitro-5-glyceryl-methylaniline was recovered in the skin wash after 30 min of exposure. After 24 hours, 0.20% of the applied dose was retained in the stratum corneum; those amounts are not considered to be percutaneously absorbed. At the end of the experiment, 0.11% of the dose applied was found in the skin (epidermis + dermis).

The mean penetration of 2-nitro-5-glyceryl-methylaniline into the receptor fluid after 24 hours was 0.27 µgeq/cm², representing 0.13% of the dose applied. The mean maximal flux for the absorption of 2-nitro-5-glyceryl-methylaniline through human skin was 0.0183 µgeq/cm² per hour. No clear difference in penetration of 2-nitro-5-glyceryl-methylaniline was observed between the four donors.

The mean total absorption (dermal delivery), defined as the compound-related radioactivity present in the receptor fluid, the receptor compartment wash and the skin (excluding tape strips), was 0.51 µgeq/cm², corresponding to 0.24% of the dose applied.

The results obtained are summarised in the table below:

	Amount recovered (µgeq/cm²)									
Cell number	**R1**	**R2**	**R3**	**R4**	**R5**	**R6**	**R7**	**R8**	**mean**	**SD**
Donor	**1**	**2**	**3**	**4**	**1**	**3**	**4**	**2**		
Skin wash	195.3	192.9	221.0	192.3	204.0	199.2	218.2	204.3	**203.4**	**11.0**
Cotton swabs	0.04	0.09	0.06	0.07	0.05	0.05	0.04	0.05	**0.06**	**0.02**
Donor compartment	0.00	0.01	0.00	0.01	0.01	0.00	0.01	0.01	**0.01**	**0.00**
Dislodgeable dose (1)	195.4	193.0	221.1	192.4	204.1	199.2	218.2	204.3	**203.5**	**11.0**
Tape strips	0.28	0.56	0.54	0.42	0.31	0.50	0.36	0.42	**0.42**	**0.10**
Unabsorbed dose (2)	195.6	193.6	221.6	192.8	204.4	199.7	218.6	204.7	**203.9**	**11.0**
Receptor fluid and wash	0.05	0.11	0.47	0.32	0.19	0.67	0.30	0.04	**0.27**	**0.22**
Skin	0.13	0.34	0.30	0.27	0.19	0.28	0.17	0.22	**0.24**	**0.07**
Total absorption	**0.18**	**0.45**	**0.77**	**0.60**	**0.38**	**0.94**	**0.46**	**0.27**	**0.51**	**0.25**
Total recovery	195.8	194.0	222.4	193.4	204.8	200.7	219.0	205.0	**204.4**	**11.1**

	Amount recovered (%)									
Cell number	**R1**	**R2**	**R3**	**R4**	**R5**	**R6**	**R7**	**R8**	**mean**	**SD**
Donor	**1**	**2**	**3**	**4**	**1**	**3**	**4**	**2**		
Skin wash	92.8	92.4	107.5	91.4	97.2	94.8	105.1	97.2	**97.3**	**6.0**
Cotton swabs	0.021	0.044	0.030	0.032	0.025	.026	0.018	0.023	**0.027**	**0.008**
Donor compartment	0.002	0.005	0.001	0.005	0.006	0.001	0.003	0.004	**0.003**	**0.002**
Dislodgeable dose (1)	92.8	92.4	107.5	91.5	97.2	94.9	105.1	97.2	**97.3**	**6.0**
Tape strips	0.13	0.27	0.26	0.20	0.15	0.24	0.17	0.20	**0.20**	**0.05**
Unabsorbed dose (2)	93.0	92.7	107.8	91.7	97.4	95.1	105.2	97.4	**97.5**	**6.0**
Receptor fluid and wash	0.02	0.05	0.23	0.15	0.09	0.32	0.14	0.02	**0.13**	**0.10**
Skin	0.06	0.16	0.15	0.13	0.09	0.13	0.08	0.11	**0.11**	**0.03**
Total absorption	**0.09**	**0.22**	**0.37**	**0.28**	**0.18**	**0.45**	**0.22**	**0.13**	**0.24**	**0.12**
Total recovery	93.0	92.9	108.2	92.0	97.6	95.5	105.5	97.5	**97.8**	**6.0**

(1) Amount in skin wash, cotton swabs, and donor compartment wash
(2) Amount in dislodgeable dose and tape strips
(3) Amount in receptor fluid, receptor compartment wash and the skin (excluding tape strips)

Conclusion
Under the experimental conditions, the study authors concluded that the mean total absorption (= amount present in the receptor fluid, receptor compartment wash and the skin, excluding tape strips) was 0.51 µgeq/cm² (0.24% of the applied dose) under non-oxidative conditions.

Ref.: 13

Comment

Too few chambers were used. The mean + 2 standard deviations (1.01 µg/cm² (0.51 + 2 x 0.25)) should be used for the calculation of the Margin of Safety.

3.3.5. Repeated dose toxicity

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

Taken from opinion SCCNFP/0688/03

Guideline:	OECD n° 407
Species:	Sprague Dawley rat - Crl CD (SD) BR
Group size:	10 males and 10 females
Substance:	B60 suspended in 0.5% carboxymethylcellulose
Batch:	op 6
Purity:	99.9 %
Dose levels:	0, 100, 300 and 1000 mg/kg bw/day in a volume of 5 ml/kg
Exposure:	29 or 30 days
GLP:	in compliance
Study period:	14 February 1990 – 16 March 1990

The test substance, suspended in 0.5% carboxymethylcellulose, was administered at 100, 300 and 1000 mg/kg bw/day daily for 29 or 30 days by gavage. The control group received the vehicle alone.
All animals were observed twice daily for mortality and once daily for clinical signs. Body weight and food consumption were recorded at weekly intervals. Ophthalmoscopic examinations were performed before the start of treatment and during week 4. Blood samples were taken from all animals during week 4 for haematological and clinical chemistry investigations. Urine samples were collected during week 4 from all animals. At autopsy, organ weights were recorded and the main organs were examined macroscopically and histologically. The eye was not examined histologically.

Results
No mortalities occurred due to the test substance. One mortality in the male high dose group was considered to be due to a gavage error. Body weights and food consumption of treated animals were comparable to controls. Hypersalivation was noted in 2/10 males and 5/10 females in the group given 1000 mg/kg bw/day. Due to the nature of the compound, the urine of the treated groups was coloured yellow throughout the test period and a yellowish staining of the fur was noted in rats given B60 at 300 or 1000 mg/kg bw/day, from day 2 onwards.
Ophthalmoscopic examination revealed a treatment related bilateral coloration of the fundus oculi in 7/10 males and 4/10 females from the 300 mg/kg bw/day group and 9/9 males and 9/10 females from the 1000 mg/kg bw/day groups. Haematological and urinalysis results did not show any treatment related changes. At termination of the experiment, absolute and relative liver weights were slightly increased at the top dose of 1000 mg/kg bw/day, but there were no related histopathological findings. All other macroscopic and microscopic observations revealed no abnormalities related to the treatment.
The authors considered that the discoloration of the eye was due to the staining properties of the dye and was not of toxicological significance. They therefore concluded that 300 mg/kg bw/day was the "No Toxic Effect Level".
On the basis of ophthalmoscopic examination 100 mg/kg bw/day should therefore be considered as the NOAEL.

Ref.: 5 (subm I)

Comment
Because of the coloration of the fundus oculi, the SCCP considers the NOEL to be 100 mg/kg bw per day.

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

Guideline:	OECD 408
Species/strain:	Sprague-Dawley rats
Group size:	40 per sex (10 per sex and per dose)
Test substance:	IMEXINE FT (1-methylamino-2-nitro-5-(2,3-dihydroxypropyloxy)-benzene)
Batch:	Op. T107
Purity:	98.3%
Dose:	0, 50, 200 or 800 mg/kg/day
Route:	oral in 0.5% aqueous methylcellulose
Exposure:	daily for 13 weeks
GLP:	in compliance
Study period:	12 April 1994 – 15 July 1994

Three groups of 10 rats/sex received B060 daily by oral gavage at doses of 50, 200 or 800 mg/kg/day in 0.5% aqueous methylcellulose for 13 weeks. A control group of 10 rats/sex received the vehicle alone. Animals were observed twice daily for mortality/morbidity and once daily for clinical signs. Body weight and food and water consumption were recorded. Ophthalmologic evaluations on control and high-dose animals were performed before the exposure; all animals were examined during week 13. Haematology, clinical chemistry and urinalysis evaluations were performed on week 13. Any animal killed prematurely or found dead during the exposure was subjected to a macroscopic examination and tissues were preserved for microscopic evaluation. Animals were killed at the end of exposure, grossly examined and selected organs were weighed. All animals were subjected to a complete macroscopic examination. All macroscopic lesions and required tissues from animals in the control and high-dose groups were evaluated microscopically; macroscopic lesions and the pancreas, lungs, liver and kidneys were evaluated in all animals of the low and intermediate dose groups. Additionally, the adrenals of all males in the low and intermediate dose groups were evaluated.

Results
Mortality occurred in 7/10 males and 6/10 females at the group of 800 mg/kg/day. Histopathology of these animals revealed vacuolated pancreatic cells, tubular nephrosis and vacuolated renal tubular cells. Clinical signs consisted of ptyalism at 200 and 800 mg/kg/day, soiled litter at 800 mg/kg/day and signs of poor clinical condition, including piloerection, hunched back, hypokinesia, swollen or hard abdomen, emaciation, dehydration and/or half-closed eyes in animals that died and in also surviving animals at 800 mg/kg/day. At all doses yellowish discolouration of urine, tail and body extremities indicate systemic exposure. Lower mean body weight gain in males and higher food consumption in both sexes were observed at 800 mg/kg/day. Body weight loss was limited to males at 800 mg/kg/day from week 8 to the end of the exposure. Bilateral opacity of the lens was noted in surviving animals at 800 mg/kg/day.
Bilateral yellowish colouration of the fundus oculi, attributed to the staining properties of the test substance, was noted in all surviving animals given 200 or 800 mg/kg/day.
Several clinical chemistry parameters were affected in surviving animals at the exposure level of 800 mg/kg/day. Higher absolute and relative kidney, adrenal and liver weights at 800 mg/kg/day were reported. Lower absolute and relative thymus and spleen weights at this dose level were attributed to stress and poor clinical condition. Enlarged or grey/green kidneys at 800 mg/kg/day correlated with tubular nephrosis and/or vacuolated tubular epithelium. Enlarged liver noted at 800 mg/kg/day correlated with higher organ weights, but had no microscopic changes. Enlarged adrenals in one male exposed to 800 mg/kg/day correlated with cortical cell vacuolation. Small thymuses and spleens at 800 mg/kg/day correlated with lower organ weights and lymphoid depletion observed microscopically. Blackish/brownish/reddish discolouration of the glandular stomach mucosa correlated with erosions observed microscopically in some animals. Histopathological changes reported

consisted of vacuolated Langerhans islet cells in the pancreas and renal tubular epithelial cells, and tubular nephrosis at 800 mg/kg/day.

Conclusion
The NOAEL for this study was 200 mg/kg/day.

Ref.: 5

Comment
Bilateral coloration was reported in the fundus oculi in all animals at 1000 mg/kg/ 28 days and 7/10 males and 4/10 at 300 mg/kg bw 28 days. Bilateral opacity of the lens was seen at 800 mg/kg/90 days. Discoloration of the fundus oculi was seen at 200 and 800 mg/kg bw/90 days. Therefore, the SCCP considers the NOEL to be 50 mg/kg bw per day.

3.3.5.3. Chronic (> 12 months) toxicity

No data submitted

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1 Mutagenicity / Genotoxicity *in vitro*

Bacterial reverse mutation test, study 1

Guideline:	/
Species/strain:	*S. typhimurium*, TA98, TA100, TA1535, TA1537, TA1538
Replicates:	Triplicate plates, only 1 test performed
Test substance:	B 60 dissolved in DMSO
Batch:	Batch op26
Purity:	/
Concentrations:	9 concentrations – direct plate incorporation assay With and without metabolic activation 10, 20, 50, 100, 250, 500, 1000, 2500 & 5000 µg/plate
GLP:	/

2-Nitro-5-glyceryl methylaniline has been investigated for gene mutation in *S. typhimurium* using the direct plate incorporation method, both with or without S9-mix. Liver S9 fraction from Aroclor 1254-induced rats was used as the exogenous metabolic activation system.

Results
Toxicity: no data.
With or without S9-mix: no dose related or biologically relevant increase in revertant numbers was observed, in any of the 5 *S. typhimurium* tester strains.

Conclusions
Based on the results and under the conditions of the assays performed, it was concluded that the test agent B 60 was negative in the *S. typhimurium* tester strains in the absence or presence of S9-mix

Ref.: 7 (submission I)

Comment
The study is of limited value for mutagenicity assessment, because the purity of the test substance was not given, no guidelines were followed, no quality insurance system was applied, and no independent repeat experiments were performed.

Bacterial reverse mutation test, study 2

Guideline:	EC B14
Species/strain:	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537; *Escherichia coli* WP2*uvr*A
Replicates:	triplicates in two independent experiments
Test substance:	Imexine FT
Solvent:	DMSO
Batch:	T 107
Purity:	98.3%
Concentrations:	312.5, 625, 1250, 2500 and 5000 µg/plate, without and with S9-mix
Treatment:	experiment 1: direct plate incorporation method with 48-72 h incubation, without and with S9-mix
	experiment 2: direct plate incorporation method with 48-72 h incubation, without S9-mix pre-incubation method with 60 minutes pre-incubation and 48 - 72 h incubation, with S9-mix
GLP:	in compliance
Study period:	March - September 1994

Imexine FT was investigated for the induction of gene mutations in *Salmonella typhimurium* and *Escherichia coli* (Ames test). Liver S9 fraction from Aroclor™-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the results of a preliminary toxicity test up to the prescribed maximum concentration of 5 mg/plate with TA100 both without and with S9-mix. Toxicity was evaluated on the basis of a reduction in the number of revertant colonies and/or qualitative evaluation of the bacterial background lawn. Experiment 1 and experiment 2 without S9-mix were performed with the direct plate incorporation method, experiment 2 with S9-mix with the pre-incubation method. Negative and positive controls were in accordance with the OECD guideline.

Results
Toxicity was only observed in TA1537 (1250 µg/plate and above) and in TA100 (5000 µg/plate) in experiment 1 without S9-mix. A reproducible, dose dependent increase in the number of revertants was not found for any of the strains tested both in the presence and absence of S9 metabolic activation. A two-fold increase in revertants of strain TA1537 in the absence of S9-mix was not reproduced in the second experiment. An increase in revertants of strain TA1537 in the presence of S9-mix was not considered to be significant because the values achieved in the first test were within the historical control range of the laboratory and those of the second test were due to one plate which showed an aberrantly high value.

Conclusion
Under the experimental conditions used Imexine FT was not genotoxic (mutagenic) in the gene mutation tests in bacteria both in the absence and the presence of S9 metabolic activation.

Ref.: 6

Mouse Lymphoma Assay (*tk* locus)

Guideline:	OECD 476
Cells:	L5178Y $tk^{+/-}$ mouse lymphoma cells
Replicates:	single cultures in two independent experiments
Test substance:	2-nitro-5-glyceryl methylaniline (Imexine FT)
Solvent:	DMSO
Batch:	01221172
Purity:	98.4%

Concentrations:	Experiment 1: 500, 1000, 1200, 1400, 1600, 1800, 2000, 2200 and 2420 µg/ml without S9-mix 250, 500, 1000, 1200, 1400, 1600, 1800 and 2000 µg/ml with S9-mix
	Experiment 2: 250, 500, 1000, 1200, 1400, 1600, 1800, 2000, 2200 and 2420 µg/ml without S9-mix 250, 500, 1000, 1200, 1400, 1600, 1800, 2000, 2200 and 2420 µg/ml with S9-mix
Treatment	3 h both without and with S9-mix; expression period 2 days and a selection period of 11 days
GLP:	in compliance
Study period:	September - November 2004

2-nitro-5-glyceryl methylaniline was assayed for mutations at the *tk* locus of mouse lymphoma cells both in the absence and presence of metabolic activation. Test concentrations were based on the results of a cytotoxicity range-finding experiment measuring reduction in relative total growth compared to the concurrent vehicle control cell cultures. In the main test, cells were treated for 3 h followed by an expression period of 2 days to fix the DNA damage into a stable *tk* mutation. Liver S9 fraction from Arochlor 1254-induced rats was used as exogenous metabolic activation system. Toxicity was measured as percentage relative total growth (relative suspension growth of the cells over the 2-day expression period multiplied by the relative cloning efficiency at the time of selection). To discriminate between large (indicative for mutagenic effects) and small colonies (indicative for a clastogenic effect), colony sizing was performed. Negative and positive controls were in accordance with the OECD guideline.

Results
The appropriate level of toxicity (10-20% survival after the highest dose) was almost reached (22-26%) in the absence of S9-mix and reached in the presence of S9-mix. Biological relevant increases in mutant frequency were observed in the absence of S9 at the two highest concentrations analysed in experiment 1 (2200 and 2420 µg/ml) and at the highest concentration (2420 µg/ml) evaluated in experiment 2. In the presence of S9-mix, obvious biological relevant and concentration-dependent increases in mutant frequency were observed in both experiments. Although increases in both small and large colony mutant frequencies were observed, there appeared to be a greater increase in the proportion of small-colony mutants.

Conclusion
Under the experimental conditions used 2-nitro-5-glyceryl methylaniline treatment did result in an increase of the mutant frequency at the *tk* locus of mouse lymphoma cells and, consequently, 2-nitro-5-glyceryl methylaniline is mutagenic in the mouse lymphoma assay.
Ref.: 7

Gene mutation assay in mammalian cells (*hprt* locus)

Guideline:	OECD 476
Cells:	L5178Y $tk^{+/-}$ mouse lymphoma cells
Replicates:	duplicate cultures in 3 independent experiments
Test substance:	2-nitro-5-glyceryl methylaniline (Imexine FT)
Solvent:	DMSO
Batch:	0122117
Purity:	98.4 %
Concentrations:	Experiment 1: 400, 800, 1200, 1400, 1600, 1800 and 2000 µg/ml without S9-mix 400, 800, 1200, 1400, 1600 and 1800 µg/ml with S9-mix
	Experiment 2: 400, 1200, 1600, 2000 and 2200 µg/ml without S9-mix

	400, 800, 1400, 1600, 1800, 2000 and 2200 µg/ml with S9-mix
	Experiment 3: 400, 800, 1400, 1600, 1800 and 2000 µg/ml with S9-mix
Treatment	3 h both without and with S9-mix; expression period 7 days and a selection period of 12-13 days
GLP:	in compliance
Study period:	July – September 2005

2-nitro-5-glyceryl methylaniline was assayed for mutations at the *hprt* locus of mouse lymphoma cells both in the absence and presence of metabolic activation. Test concentrations were based on the results of a cytotoxicity range-finding experiment measuring reduction in relative survival compared to the concurrent vehicle control cell cultures. In the main test, performed according the microtitre^{R} fluctuation technique, cells were treated for 3 h followed by an expression period of 7 days to fix the DNA damage into a stable *hprt* mutation. Liver S9 fraction from Arochlor 1254-induced rats was used as exogenous metabolic activation system. Toxicity was measured as percentage relative survival. Negative and positive controls were in accordance with the OECD guideline.

Results
In experiment 1 and experiment 2 with S9-mix the appropriate level of toxicity (10-20% survival after the highest dose) was reached. In experiment 2 without S9-mix and in experiment 3 survival was higher than the appropriate level.
In experiment 1 and 2 in the absence of S9-mix, a biological relevant increases in mutant frequency was not observed following treatment with 2-nitro-5-glyceryl methylaniline. A weak, trend was observed in the second experiment but no significant increases in mutant frequency were observed at any dose tested and the effect was not reproduced between experiments, therefore this increase was not considered biologically relevant.
In the presence of S9-mix when tested up to toxic doses, statistically significant increases in mutant frequency were observed at two intermediate doses (1200 and 1400 µg/ml) in experiment 1 and at the highest dose (2200 µg/ml) analysed in experiment 2. Linear trends were observed in both experiments. In experiment 1, the mean mutant frequency values observed at both concentrations where a statistically significant response was observed and those at 800 and 1800 µg/ml exceeded the upper limit of the historical control range. In experiment 3, performed with S9-mix to confirm the results from experiment 1 and 2, a biological relevant increase in mutant frequency was not observed. Overall, it is considered that the statistically significant increases in mutant frequency observed in experiments 1 and 2 were not large and were not reproduced in confirmatory experiment 3.

Conclusion
It was concluded that 2-nitro-5-glyceryl methylaniline (B060) did not induce mutations at the *hprt* locus of L5178Y mouse lymphoma cells in two independent experiments in the absence of rat liver S9 mix when tested under the conditions employed in this study. In the presence of S9 mix in the same test system, 2-nitro-5-glyceryl methylaniline showed evidence of increased mutant frequency in two experiments but these effects were not reproduced in a third experiment. The evaluation criteria for a positive result were not fulfilled and these increases were considered of little or no biological relevance.

Ref.: 8

Comment
Two of three independent tests in the presence of S-9 mix were positive, with a concentration-dependent effect. Given the positive result in the mouse lymphoma tk$^{+/-}$ test, the SCCP considers this test inconclusive/equivocal. The possibility that 2-nito-5-glyceryl methylaniline has the potential to induce gene mutations in cultured mammalian cells cannot be excluded based on this test/the two mammalian cell gene mutation tests.

In vitro mammalian chromosomal aberration test, study 1

Guideline:	/
Species/strain:	Chinese hamster ovary (CHO) cells
Replicates:	Duplicate cultures but no independent experiment
Test substance:	2-Nitro-5-glyceryl methylaniline in DMSO solution
Batch:	/
Purity:	/
Concentrations:	Preliminary dose range finding study: No raw data given Test without S9-mix: 0, 0.5, 1, 2 and 4 mg/ml Test with S9-mix: 0, 0.5, 1, 2 and 4 mg/ml
GLP:	/

2-Nitro-5-glyceryl methylaniline has been investigated for induction of chromosomal aberrations in CHO cells. 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. Samples were exposed during 1 hour with or without S9. Cultures were kept for 19 hours before harvest.

Results
For toxicity no raw data were given. While both with and without S9-mix a slight dose dependent trend of aberrations was found, no statistics have been used to evaluate the incidence of aberrant cells. Significant increase in the aberration rate was observed as compared to the corresponding solvent control, mainly in the absence of activation.

Conclusions
The results suggested a clear clastogenic effect.

Ref.: 8 (submission I)

Comments
No statistics were performed. The treatment time was short (1 h), the exposure and expression period are inadequately selected, the test substances was not characterised (batch and purity not specified). As the assay was not performed according to modern standard strategies and guidelines, the results are considered inadequate.

In vitro mammalian chromosomal aberration test, study 2

Guideline:	OECD 473, EC B10
Species/strain:	Chinese hamster ovary (CHO) cells
Replicates:	Duplicate cultures, single experiment
Test substance:	2-nitro-5-glyceryl methylaniline in DMSO Imexine FT
Batch:	Batch No 0503126
Purity:	/
Concentrations:	23 - 2420 µg/ml with and without metabolic activation
GLP:	in compliance

2-nitro-5-glyceryl methylaniline has been investigated for induction of chromosomal aberrations in CHO cells. Liver S9 fraction from Aroclor 1254-induced rats was used as the exogenous metabolic activation system.
The analysed test concentrations were selected from the range of treatment concentrations based on the cytotoxicity observed. With respect to the molecular weight of 2-nitro-5-glyceryl methylaniline, the maximum concentration tested was 2420 µg/ml (corresponding to 10 mM).

	Exposure period	*Recovery*	*Preparation interval*	*Concentrations µg/ml*
Experiment without S9-mix	3 hours	17 hours	20 hours	407, 1694, 2420
Experiment with S9-mix	3 hours	17 hours	20 hours	1186, 1694, 2420

Results
On the post-treatment medium, no marked influence of the pH or osmolarity was noted as compared to the concurrent vehicle controls.
A 68 % reduction of the mitotic index was noted in the absence of activation at the top dose level (2420 µg/ml). With S9, at the top dose the mitotic index reduction was 31% of the control.
Without activation system a significant and biologically relevant increase in the number of cells with structural chromosomal aberrations was noted at the top dose of 2420 µg/ml (2420 µg/ml 14 %). With activation system a statistically and/or biologically significant dose-dependent relevant increase in the number of aberrant cells was observed as compared to the corresponding solvent control at the top dose of 2420 µg/ml (2420 µg/ml 13.5%).
Taken into account that no specific positive control agent has been used in this assay, and that only metaphases with 19 -23 chromosomes were considered for scoring, polyploidy means a number of chromosome > 23 or endoreduplication. Without activation system a relevant increase in the number of polyploid metaphases was recorded at the top dose. The number of cells displaying numerical aberrations was of 3.8 % in the control and 7.8 % at the top dose. With activation system a relevant increase in the number of polyploid metaphases was recorded at the top dose. The number of cells displaying numerical aberrations was of 4.8 % in the control and 10.3 % at the top dose. Moreover, in one replicate of the intermediate dose, 8 % of polyploid cells were noted. In addition, many endoreduplicated cells were observed in the presence of the activation system.

Conclusions
IMEXINE FT induced chromosomal aberrations in Chinese hamster ovary cells in the absence and presence of metabolic activation, under the conditions of this test. In addition indication of aneugenicity was noted under both conditions at the top dose levels.

Ref.: 11 (submission I)

In vitro unscheduled DNA synthesis test in human HeLa cells

Guideline:	/
Cells:	Hela S3 cells
Replicates:	triplicates in two independent experiments
Test substance:	3389 Pan
Solvent:	DMSO
Batch:	BL VII P 108
Purity:	/
Concentrations:	Experiment 1: 0.156, 0.313, 0.625, 1.25 and 2.50 mg/ml without S9-mix 0.313, 0.625, 1.25, 2.50 and 5.0 mg/ml with S9-mix
Treatment	3 h treatment
GLP:	in compliance
Study period:	April – December 1985

3389 Pan was investigated for the induction of unscheduled DNA synthesis (UDS) in human Hela cells. Test concentrations were based on the results of a cytotoxicity assay on cell

viability, cell detachment and signs of gross toxicity. Cells were treated for 3h with 5µCi ^{3}H-thymidine and then progressed for autoradiography. The induction of UDS has been measured by liquid scintillation counting. Liver S9 fraction from phenobarbitone/β-naphthoflavone-induced rats was used as the exogenous metabolic activation system. 4-nitroquinoline-N-oxide and benzo[a]pyrene were used as positive control.

Results
In the absence of metabolic activation, the incorporation of ^{3}H-thymidine was similar to that of negative controls at the three lowest treatment levels. At 1.25 and 2.50 mg/ml, incorporation levels declined to approximately 90% and 50% of the control levels, respectively. In the presence of metabolic activation, the incorporation of ^{3}H-thymidine decreased in a dose-dependent manner. At 5.00 mg/ml, incorporation was approximately 30% of control levels.

Conclusion
Under the experimental conditions used 3389 Pan treatment did not result in an increase in unscheduled DNA synthesis and, consequently, 3389 Pan is not genotoxic in this UDS test in human Hela cells.

Ref.: 9

Comment
According to the modern standard strategies and guidelines, this assay is unsuitable for evaluation. UDS measured by a liquid scintillation counter is not an established test system and may not be very sensitive. Therefore, the results have limited value in assessing the genotoxicity of the test substance. The purity of the test substance is not reported.
Moreover, the report contains a number of inconsistencies and omissions:

- There are two summaries: "summary and conclusion" on the back side of the front page and "1 summary" on page 1 of the report.
- Both in the summaries and in the description of the "4.3 main assay (UDS)" it is stated that the highest concentration to be tested was 2.50 mg/ml in the presence and absence of metabolic activation, while the data table for UDS in the presence of metabolic activation indicates that the highest concentration tested was 5.00 mg/ml.
- The numbering of the tables containing the assay results does not correspond to the description in the results section.
- detailed results are not given for a second experiment described in the results section.

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Mammalian erythrocyte micronucleus test, study 1

Guideline:	/
Species:	Albino Swiss mouse
Group sizes:	10 males dosed, 5 control and 6 test animals evaluated
Substance:	B60 dissolved in DMSO
Batch:	DG1
Purity:	/
Dose levels:	0, 350, 450 and 550 mg/kg in a volume of 10 ml/kg
Route:	intraperitoneal injection
Administration:	2 intraperitoneal injections, 24 hours apart
Sacrifice times:	after 24, 48 and 72 hours
GLP:	/

2-Nitro-5-glyceryl methylaniline has been investigated for induction of micronuclei in the bone marrow cells of male or female mice. Dose levels were determined on the basis of the results of a preliminary dose-range finding study having showed that LD50 is 650 mg/kg bw. Negative and positive controls were in accordance with the OECD guideline.

2-Nitro-5-glyceryl methylaniline dissolved in DMSO was administrated by 2 single intraperitoneal injections with 24 h interval. Male mice: 0, 350, 450 and 550 mg/kg. One sacrifice time was selected: 6 hours after last dosing. Bone marrow smears were obtained from the positive control group 24 after dosing.
A total of at least 2000 erythrocytes were examined from each animal; the incidence of micronucleated erythrocytes and the ratio of polychromatic erythrocytes to normochromatic erythrocytes were calculated.

Results
Toxic effects such as passivity, dyspnea and ataxia were observed in all dosage groups. No statistically significant or biologically relevant increase in the incidence of micronucleated polychromatic cells over the concurrent vehicle control values were observed at any sampling times. No significant variations in the ratio of normochromatic to polychromatic erythrocytes, which would have indicated that the bone marrow was reached by the test material and or toxicity of the latter, were noted.

Conclusions
Under the test conditions, 2-nitro-5-glyceryl methylaniline did not induce micronuclei in the bone marrow of mice.

Ref.: 9 (submission I)

Comments
The test did not conform to OECD guidelines or GLP conditions. Only one experiment was performed, and the number of animals per dose was insufficient. The test is considered inadequate.

Mammalian erythrocyte micronucleus test, study 2

Guideline:	OECD 474 (1997)
Species:	Swiss Ico: OF1(IOPS caw) mice
Group size:	5 males and 5 females
Test substance:	Imexine FT (2-nitro-5-glyceryl methylaniline) in 1 % methylcellulose
Batch:	0504494
Purity:	/
Dose levels:	0, 500, 1000 & 2000 mg/kg bw
Administration:	2 intragastric gavages on 2 consecutive days (24-hours interval)
Sacrifice times:	24 hours after the last dosing.
GLP:	in compliance
Study period:	12 March – 20 April 2001

Imexine FT 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 no toxic effects were seen. The substance was administered by two single intragastric gavages at 24-hours interval and the groups of animals sacrificed 24 hours after the last administration. Negative and positive controls were in accordance with the OECD guideline. 2-nitro-5-glyceryl methylaniline in 1 % methylcellulose, batch 0504494 (purity not stated was administered by 2 single oral doses at 24-hours intervals.
One sacrifice time was selected: 24 h after the last oral administration. Bone marrow smears were obtained from the positive control group 24 hours after dosing.
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
No statistically significant or biologically relevant increase in the incidence of micronucleated polychromatic cells over the concurrent vehicle control values were observed for any dosage

groups. No significant reduction in the PCE/NCE ratio was observed in any of the dosage groups of mice treated with Arianor Straw Yellow.

Conclusions
Under the conditions of the test it can be concluded that Imexine FT in doses at which no significant variation in the PCE/NCE ratio was observed, does not induce statistically significant increase in the frequency of PCE. The negative and positive controls gave the expected results. However, it should be noted that a trend to a dose response was observed and that there is large inter-individual variations in the individual values.

Ref.: 12 (submission I)

Mouse bone marrow micronucleus test, study 3

Guideline:	OECD 474
Species/strain:	Swiss Ico; OF1 (IOPS Caw) mice
Group size:	5 mice/sex/dose group
Test substance:	Imexine FT
Batch no:	0504494
Purity:	98%
Dose level:	500, 1000 and 2000 mg/kg bw
Route:	oral
Treatment:	2 applications 24 h apart
Vehicle:	1% aqueous methylcellulose
Sacrifice times:	24 h after the last treatment.
GLP:	in compliance
Study period:	February – May 2001

Imexine FT has been investigated for the induction of micronuclei in bone marrow cells of mice. Test concentrations were based on the results of a preliminary toxicity test in male and female mice on clinical signs and mortality for a period of 48 h. In the main experiment mice received orally two treatments of 0, 500, 1000 and 2000 mg/kg bw 24 h apart. Bone marrow cells were collected 24 h after the last dosing. Toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic and normochromatic erythrocytes (PCE/NCE). Bone marrow preparations were stained with Giemsa and examined microscopically for the PCE/NCE ratio and micronuclei. Negative and positive controls were in accordance with the OECD guideline.

Results
Both in the preliminary study and the main experiment, no mortality and no clinical signs were observed. Treatment with Imexine FT did not result in decreased PCE/NCE ratios compared to the untreated controls. Biological relevant increases in the number of micronucleated PCEs compared to the concurrent vehicle controls were not found at any dose tested.

Conclusion
Under the experimental conditions used Imexine FT did not induce micronuclei in bone marrow cells of treated mice and, consequently, Imexine FT was not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of mice.

Ref.: 10

Comment
Since treatment with Imexine FT did not result in a decrease of the PCE/NCE ratio and there were no other indications of bone marrow exposure, the test is of limited value.

Mouse bone marrow micronucleus test, study 4

Guideline:	OECD 474 (1997)
Species/strain:	Crl:CD-1®(ICR)BR mice
Group size:	5 mice/sex/dose group
Test substance:	2-nitro-5-glyceryl methylaniline
Batch no:	0122117
Purity:	98.4 %
Dose level:	250, 500 and 1000 mg/kg bw
Route:	oral gavage
Vehicle:	0.5 % methylcellulose
Sacrifice times:	24 h after treatment for all concentrations, 48 h for the control and highest dose group only.
GLP:	in compliance
Study period:	November 2004 - June 2005

2-nitro-5-glyceryl methylaniline has been investigated for the induction of micronuclei in bone marrow cells of mice. Test concentrations were based on the results of a dose range-finding study in male and female mice on toxic signs and mortality. In the main experiment mice were exposed by gavage to single doses of 0, 250, 500 and 1000 mg/kg bw. Mice were examined immediately and 1 h after dosing and at least daily for the duration of the experiment for signs of clinical toxicity and mortality. Bone marrow cells were collected 24 h or 48 h (control and high dose only) after dosing. Toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic and normochromatic erythrocytes (PCE/NCE). An additional satellite group of 6 male and 6 female mice, treated with 1000 mg/kg bw, was included for determination of plasma concentrations of the test article. Blood was collected at 1 and 4h after dosing (3 mice/sex/per timepoint). Bone marrow preparations were stained with acridine orange and examined microscopically for the PCE/NCE ratio and micronuclei. Negative and positive controls were in accordance with the OECD guideline.

Results
In the range-finding study clinical signs were noted in animals given 1000 and 2000 mg/kg 2-nitro-5-glyceryl methylaniline from 1st hour post-dosing upwards, including hypoactivity, irregular respiration, squinted eyes, and orange discoloration of ears and tail. On day 1 after treatment, no more signs were observed in the animals given 1000 mg/kg, while these observations persisted or increased in the high dose group. Based on these results, the maximum tolerated dose was estimated to be 1000 mg/kg and this dose level was used as the high dose in the main study.
No mortality occurred during the study. All animals in all doses groups showed hypoactivity, discoloured ears, tails and limbs and squinted eyes at 1 hour post-dosing. On day 1 after treatment, these signs had disappeared.
Treatment with 2-nitro-5-glyceryl methylaniline did not result in decreased PCE/NCE ratios compared to the untreated controls. However, plasma analysis confirmed the systemic exposure of the test animals to the compound (mean C_{max} 54.3 ± 15.3 µg/ml at 1 h post dosing in males and females given 1000 mg/kg).
Biological relevant increases in the number of micronucleated bone marrow cells compared to the concurrent vehicle controls were not found at any dose tested, neither 24 nor 48 h after treatment.

Conclusion
Under the experimental conditions used 2-nitro-5-glyceryl methylaniline did not induce micronuclei in bone marrow cells of treated mice and, consequently, 2-nitro-5-glyceryl methylaniline was not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of mice.

Ref.: 11

3.3.7. Carcinogenicity

No data submitted

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

No data submitted

3.3.8.2. Teratogenicity

Guideline:	OECD 414
Species/strain:	Sprague-Dawley rats
Group size:	mated rats in four groups (n=36, n=33, n=40, n=34)
Test substance:	IMEXINE FT
Batch:	Op. 8
Purity:	99.9%
Dose:	0, 100, 300, 1000 mg/kg/day
Route:	oral gavage
Vehicle:	0.5% aqueous carboxymethylcellulose
Exposure:	from day 6 through day 15 of gestation
GLP:	in compliance
Date:	23 October 1990 – 17 January 1991

Three groups of mated rats received B060 by oral gavage at doses of 100 (n=33), 300 (n=40) or 1000 mg/kg/day (n=34) in 0.5% aqueous carboxymethylcellulose from day 6 through day 15 of gestation. A control group of 36 mated rats received the vehicle only. The day of mating was designated as day 0 of gestation. Animals were observed twice daily for mortality/morbidity. Clinical signs were checked once daily. Food consumption and body weight were recorded. On day 20 of gestation, the animals were killed and examined macroscopically. Foetuses were removed by Caesarean section. The following litter parameters were recorded: number of corpora lutea, number of implantation sites, number and distribution of early and late resorptions, and number and distribution of dead and live foetuses. Foetuses were weighed, sexed and examined for possible external abnormalities. The first twenty litters of each group were examined for soft tissue and skeletal examinations.

Results
One 300 mg/kg/day female was found dead on day 17 as a result of gavage accident. At 1000 mg/kg/day, one female showed reddish nasal discharge and piloerection on days 9 and 10 and was sacrificed moribund on day 10. The stomach of this animal had ulcerated foci; yellow discolouration was present in the stomach, liver, skin, and kidneys. Lemon-coloured urine was observed in almost all treated animals from day 7 to day 16. No abortions were observed. Maternal body weight gain and food consumption at 1000 mg/kg/day were slightly lower than control group values; however, these changes were not statistically significant. No other compound-related findings were observed in dams at any dose level.
One dead foetus and an increase in the number of foetuses with unossified 4^{th} metacarpals were observed at 1000 mg/kg/day. No other compound-related anomalies or malformations of toxicological significance were observed.

Conclusion
The NOAEL for materno-toxicity for this study was 300 mg/kg/day, while the NOAEL for embryo-toxicity/teratogenicity was 1000 mg/kg/day.

Ref.: 12

Conclusion from previous opinion
Dams: no treatment related mortalities were observed. One mortality in the 300 mg/kg/day group was attributed to a gavaging error. All treated animals presented lemon coloured urine from day 7 to day 16. One female (1000 mg/kg/day) was culled following observation of a reddish nasal discharge and piloerection on days 9 and 10 post-coitum. At necropsy, most organs of the culled dam were stained yellow and the stomach showed some ulcerated foci. No abortions were reported.
Maternal body weight gain and food consumption were slightly reduced in the highest dose group compared to the control group. No abnormalities were recorded at necropsy. No changes were noted in the other treated groups.

Foetuses: Litter parameters were comparable between the control and treated groups. External examination revealed no treatment-related foetal malformations. No soft tissue malformations were observed. The only skeletal malformation noted was the number of foetuses from the 1000 mg/kg bw group with a delayed ossification of the fourth metacarpus when compared to controls. This observation was considered by the authors to be related to the slight maternotoxicity seen in this group.

The study authors concluded that, at 300 mg/kg bw/day, there was no evidence of maternal toxicity, embryotoxicity or teratogenicity, whereas the 1000 mg/kg bw/day dose level was slightly maternotoxic but neither embryotoxic nor teratogenic.

Comment
The study was re-assessed and the conclusion from the previous opinion was confirmed.

3.3.9. Toxicokinetics

No data submitted

3.3.10. Photo-induced toxicity

No data submitted

3.3.11. Human data

No data submitted

3.3.12. Special investigations

No data submitted

3.3.13. Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

Not applicable

3.3.14. Discussion

Physico-chemical properties
2-Nitro-5-glyceryl methylaniline is used in semi permanent hair colouring products at a maximum concentration of 1%. The formulation is applied as such without any further

dilution. 2-Nitro-5-glyceryl methylaniline is a secondary amine, and thus, prone to nitrosation. It should not be used in combination with nitrosating agents. The nitrosamine content should be < 50 ppb. The nitrosamine content in the test materials was not reported. The stability of 2-Nitro-5-glyceryl methylaniline in the marketed products was not reported.

General toxicity
The maximal non-lethal dose of 2-nitro-5-glyceryl methylaniline was higher than 1000 mg/kg but lower than 2000 mg/kg after a single oral administration in fasted rats.
In a 13-week oral study, the NOEL was set at 50 mg/kg/day.
The NOAEL for materno-toxicity was set at 300 mg/kg/day, while the NOAEL for embryo-toxicity/teratogenicity was set at 1000 mg/kg/day.

Irritation / sensitisation
The test substance was considered as non-irritant to rabbit skin when applied as a 1% suspension in 1,2-propanediol. The 1% suspension caused some slight and transient irritation of the rabbit eye.
The highest concentration tested (10%) in the LLNA was considered too low. No conclusion regarding the sensitising potential of 2-nitro-5-glyceryl methylaniline can be drawn.

Dermal absorption
Too few chambers were used. The mean + 2 standard deviations (1.01 µg/cm² (0.51 + 2 x 0.25)) should be used for the calculation of the Margin of Safety.

Mutagenicity / genotoxicity
Overall, the genotoxicity of 2-nitro-5-glyceryl methylaniline (Imexine FT) has been investigated for the three types of mutations: gene mutations, chromosome aberrations and aneuploidy.
Treatment with 2-nitro-5-glyceryl methylaniline did not result in an increase in the mutant frequency in bacteria. In the mouse lymphoma assay 2-nitro-5-glyceryl methylaniline induced an increase in the mutant frequency at the *tk* locus. Although increases in both small and large colony mutant frequencies were observed, there appeared to be a greater increase in the proportion of small-colony mutants, indicating to a clastogenic next to a mutagenic effect. In a second gene mutation assay in mammalian cells using the same cell line but the *hprt* locus as reporter gene two experiments out of 3 showed evidence of increased mutant frequency, but were considered not to be of relevance by the study authors. The SCCP considered this test inconclusive/equivocal, and the possibility that 2-nito-5-glyceryl methylaniline has the potential to induce gene mutations in cultured mammalian cells cannot be excluded based on this test/the two mammalian cell gene mutation tests.
In a poorly performed *in vitro* unscheduled DNA synthesis test 2-nitro-5-glyceryl methylaniline was negative. The putative clastogenic effect was confirmed in two poorly performed *in vitro* chromosome aberration tests.
The clastogenic effects found in the *in vitro* studies could not be confirmed in *in vivo* experiments covering the same endpoint. In four (3 poorly and one well performed) mouse bone marrow micronucleus tests, following oral and i.p. administration, 2-nitro-5-glyceryl methylaniline was negative.
However, the positive finding in one of the *in vitro* gene mutation assays remains and the second test (*hprt*-test) was not considered adequate to exclude a potential to induce gene mutations in mammalian cells. Therefore, to reach a definitive conclusion on the genotoxicity of 2-nitro-5-glyceryl methylaniline an appropriate *in vivo* test to study the induction of gene mutations has to be performed.

Carcinogenicity
No data submitted

4. CONCLUSION

The SCCP is of the opinion that the safety of 2-nitro-5-glyceryl methylaniline cannot be assessed based on the data submitted.

Before any further consideration, an appropriate *in vivo* test to study the induction of gene mutations has to be submitted.

2-Nitro-5-glyceryl methylaniline is a secondary amine, and thus, prone to nitrosation. It should not be used in combination with nitrosating agents. The nitrosamine content should be < 50 ppb.

5. MINORITY OPINION

Not applicable

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