EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate C - Public Health and Risk Assessment
C7 - Risk assessment

SCIENTIFIC COMMITTEE ON CONSUMER PRODUCTS

SCCP

Opinion on

p-Phenylenediamine

COLIPA N° A7

Adopted by the SCCP
during the 9th plenary meeting of 10 October 2006

TABLE OF CONTENTS

1. BACKGROUND

Submission I for *p*-Phenylenediamine was submitted in December 1979 by COLIPA[1,2].

Submission II for *p*-Phenylenediamine was submitted in March 1983 by COLIPA[2].

The Scientific Committee on Cosmetology (SCC) has at its 48th meeting on 4 October 1991 expressed its opinion with the conclusions:

"Para-phenylenediamine has moderate acute toxicity by the oral route and low toxicity by the dermal route. A 2.5 % solution has no significant skin or eye irritant properties. There was evidence of skin sensitization in both animal and human studies. The results of studies in patients indicate 6-11 % are sensitised to para-phenylenediamine whilst patch testing on the general population prior to hair dyeing over a 5 year period indicated less than 0.1 % give positive results. In a 90 day dermal study no effects were reported with hair dye formulations containing up to 5 % of the compound. Para-phenylenediamine has clearly been shown to have mutagenic potential in vitro, with positive results in assays for gene mutation in Salmonella and mammalian cells (mouse lymphoma assay) and also for clastogenicity in mammalian cells and UDS in hepatocytes. This activity does not appear to be expressed in vivo with negative results in a bone marrow assay for clastogenicity (micronucleus test), binding to DNA in liver and two dominant lethal assays. The in vitro activity may be related to formation of the Bandrowski's base which is very unstable. There have been no compound related effects reported during well conducted chronic studies of hair-dye formulations containing the compound by the dermal route or following oral administration of para-phenylenediamine. No compound related effects were reported in reproduction toxicity studies with either the compound alone or in hair-dye formulations. The compound is absorbed through the skin to a significant extent under occlussive dressing in the absence of oxidation (7 %); oxidation with hydrogen peroxide decreases the absorption of the compound to almost negligible levels (< 0.1 %). This is supported by studies on absorption in humans using in use conditions and radio-labelled material, only 0.34 % of the applied dose was absorbed over a 30 day period. The compound is predominantly excreted in urine and is extensively metabolised. Classification: A. Subject to restrictions on concentration and labelling already in force namely 6 % as the free base and 'can cause an allergic reaction. Do not use to dye eyelashes or eyebrows."

Submission III for p-Phenylenediamine was submitted in June 2000 by COLIPA[2].

The Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers (SCCNFP) adopted at its 19th plenary meeting on 27 February 2002 the opinion (SCCNFP/0129/99, final) with the conclusion:

"*The SCCNFP is of the opinion that the information submitted is insufficient to allow an adequate risk assessment to be carried out. Before any further consideration, additional data would be required on carcinogenicity of PPD in combination with hydrogen peroxide and*

[1]COLIPA – European Cosmetics Toiletry and Perfumery Association.

[2] According to records of COLIPA

couplers (to simulate consumer exposure) upon application to rat's skin, and on in vivo mutagenicity genotoxicity of the said combinations."

The substance is currently regulated by the Cosmetics Directive (76/768/EC), Annex III, Part 1 under entry 8 on the List of substances, which cosmetic products must not contain except subject to restrictions and conditions laid down.

Submission IV for *p*-Phenylenediamine was submitted by COLIPA in July 2005. According to this submission *p*-Phenylenediamine is used in oxidative hair dye formulations at a maximum concentration of 4.0%, which after mixing in a ratio1:1 with hydrogen peroxide prior to use, corresponds to a maximal concentration of 2.0% at application to the hair.

Submission IV 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 p-Phenylenediamine safe for use as an oxidative hair dye with a concentration on head of maximum 2.0 % taken into account the scientific data provided?*

2. *Does the SCCP recommend any further restrictions with regard to the use of p-Phenylenediamine in any oxidative hair dye formulations?*

3. OPINION

3.1. Chemical and Physical Specifications

3.1.1.	Chemical identity

3.1.1.1.	Primary name and/or INCI name

p-Phenylenediamine (INCI)
It exists also in the form of p-Phenylenediamine hydrochloride and sulfate HCl (INCI)

3.1.1.2.	Chemical names

1,4-Benzenediamine; 1,4-Diaminobenzene; 4-Aminoaniline; p-Aminoaniline

Dihydrochloride
1,4-benzenediamine dihydrochloride; 4-Aminoaniline dihydrochloride; p-Aminoaniline dihydrochloride

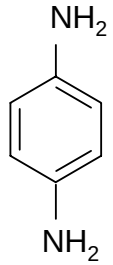
3.1.1.3. Trade names and abbreviations

PPD
CI 76 060
COLIPA n° A7

3.1.1.4. CAS / EINECS number

	free base	dihydrochloride	sulfate
CAS:	106-50-3	624-18-0	541-70-8; 25723-55-4
EINECS:	203-404-7	210-834-9	208-791-6

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_6H_8N_2$ (free base)
$C_6H_8N_2$. 2HCl (dihydrochloride)
$C_6H_8N_2$. H_2SO_4 (sulfate)

3.1.2. Physical form

White to light purple powder

3.1.3. Molecular weight

Molecular weight: 108.14 (base)
181.07 (dihydrochloride)

3.1.4. Purity, composition and substance codes

Specification

	Free Base	**Dihydrochloride**
Titre	> 98 g/100g	> 98 g/100g
Relative purity by HPLC	> 99%	> 99%

The analytical study of p-Phenylenediamine (base) was performed on five batches:

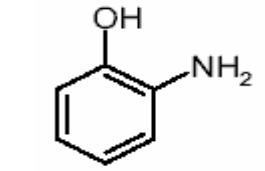
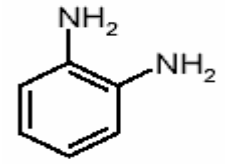
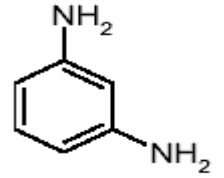
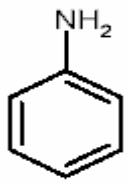
03629102 (in April 1993), 976070 (in October 1997), 1269 (in March 2004), 2G100 (in March 2004), 2L389 (in March 2004)
The analytical study of p-Phenylenediamine (dihydrochloride) was performed on one batch: 000 D002 (in April 2005)

Comparative Table of Analytical Results

Description	**Batch number**				
	03629102	**1269**	**2G100g**	**21.389**	**000 D002**
Characterisation/ Identification	IR, NMR, MS, Elemental analysis				IR, NMR, MS, Elemental analysis
HPLC purity, area %	99.6	100.2	99.0	99.6	99.3
Content determined by potentiometric titration (g/100 g)		99.8	99.8	99.8	> 99.0

3.1.5. Impurities / accompanying contaminants

Impurities

			
o-Aminophenol	**o-Phenylenediamine**	**m-Phenylenediamine**	**Aniline**
(< 500 ppm)*	(< 200 ppm)*	(< 200 ppm)*	(< 50 ppm)*

* Apparently reported as "specification limits"

The content of the possible impurities were checked in batches 03629102, 1269, 2G100, and 2L389 of the free base and found as follows:

	Batch			
	03629102	**1269**	**2G100g**	**21.389**
o-Aminophenol	No data	210 µg/g	400 µg/g	190 µg/g
o-Phenylenediamine	≤ 100 µg/g	30 µg/g	120 µg/g	< 10 µg/g
m-Phenylenediamine	< 100 µg/g	90 µg/g	140 µg/g	65 µg/g
Aniline	< 100 µg/g	50 µg/g	50 µg/g	50 µg/g

Water content: g/100 g
Loss on drying: g/100 g
Ash: <0.1 g/100 g

Solvents: < 100 ppm
Hg, Sb, As: each < 5 ppm (mg/kg)
Cd: <10 ppm
Pb: <20 ppm

3.1.6. Solubility

Free base:
Water: < 10% (w/v) at 22°C
Ethanol: < 10% (w/v) at 22°C
DMSO: > 20% (w/v) at 22°C

According to reference 1, subm. 12/2005, the solubility in water is at least 1 %.

3.1.7. Partition coefficient (Log P_{ow})

Log P_{ow}: - 0.31 (calculated Clog P v.4.2 - C. Hansch)

3.1.8. Additional physical and chemical specifications

- melting point: 139-141 °C (Merck index: 145-147 °C)
- flash point: /
- vapour pressure: < 1 mm Hg at 21°C (technical product)
- boiling point: 267 °C
- density at 20 °C: /
- viscosity: /
- pKa: /
- UV absorption spectrum λ_{max} 281.9 nm
- Refractive index at 20 °C: /

3.1.9. Stability

No data submitted

General Comments on Physico-chemical characterisation

- No data on stability in test solutions and in marketed products was submitted.
- Log $P_{ow,}$: calculated values can not be accepted as estimates of the true physical constants without justification, indicating that the reported values are realistic.
- The solubility in water is not properly characterised.
- A considerable amount of CMR 3 substances (e.g. o-aminophenol and aniline) are present as impurities in the marketed PPD.

3.2. Function and uses

p-Phenylenediamine is used as an ingredient of oxidative hair colouring products at a maximal concentration of 4.0%, which after mixing in a 1:1 ratio with hydrogen peroxide prior to use, corresponds to a maximal concentration of 2.0% at application to the hair.

3.3. Toxicological Evaluation

3.3.1.	Acute toxicity

3.3.1.1.	Acute oral toxicity

Taken from SCCNFP/0129/99, adopted in 2002

Acute toxicity has been investigated following oral, subcutaneous, intraperitoneal and topical application in a variety of species. The LD_{50} following oral administration was 80-100 mg/kg in the rat, 290 mg/kg in mice, 250 mg/kg in rabbit and 100 mg/kg in cats.

The values following subcutaneous application were 170, 200 and 100 mg/kg for rat, rabbit and dog respectively.

The intraperitoneal and topical LD_{50} values have each only been determined in the rabbits respectively. A variety of toxic effects have been reported with some variation between species. There are several reports of deliberate or accidental para-phenylenediamine poisoning in humans but no details of the amount ingested were available. The symptoms reported include oedema of the glottis and acute renal failure.

Ref.: 67, 104, 114

Guideline:	OECD 420 (2001)
Species/strain:	Sprague-Dawley rats, strain Crl: OFA (SD)
No. of animals:	9 females
Test substance:	p-phenylenediamine
Batch:	1365
Purity:	99.8 %
Dose:	25, 50, 75, or 100 mg/kg bw in deionised water
GLP:	In compliance

The test compound was given once by gavage. One animal was given 100 mg/kg bw, 2 animals received 75 mg/kg bw, one animal received 50 mg/kg bw and 5 animals received 25 mg/kg bw. The animals were observed for 14 days for mortality and clinical signs.

Results
The animal treated with 100 mg/kg bw died 90 min after dosing. 1 of 2 animals treated with 75 mg/kg died within 3 h. The animal treated with 50 mg/kg bw showed clinical signs (lachrimation, swelling of conjunctivae, gait, tremor, subdued behaviour and/or pilorection). At 25 mg/kg bw only orange traces in the bedding were seen probably due to coloured urine.

Ref.: 1, subm. 12/2005

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/0129/99, adopted in 2002

A 2.5% aqueous solution of PPD containing 0.05% sodium sulphite was mildly irritant when applied to abraded or intact rabbit skin covered by a gauze patch. The primary irritation index in a Draize rabbit test was estimated to be 0.3 out of a maximum score of 8.

Ref.: 67

3.3.2.2. Mucous membrane irritation

Taken from SCCNFP/0129/99, adopted in 2002

A 2.5% aqueous solution of PPD containing 0.05% sodium sulphite was not considered to be irritant when instilled into the rabbit eye (n=3) and then rinsed with water after 10 seconds. Minimal conjunctival irritation was seen in one animal only.

Ref.: 67

3.3.3. Skin sensitisation

Taken from SCCNFP/0129/99, adopted in 2002

Human and animal data

p-Phenylenediamine (PPD) is a very strong potential skin sensitiser and included in the European Standard Series for diagnostic patch testing of eczema patients.

The individual susceptibility to PPD seems to be under polymorphic control as acetylator phenotype may be important for metabolism and detoxification of PPD and represents a marker for determining individual susceptibility to PPD allergy. The submission contains a number of relevant references that support this statement. However, a number of important and more recent scientific publications are not included in the submission.
p-Phenylenediamine sensitises 100% of laboratory animals (both guinea pigs and mice) used in predictive allergenicity testing if the concentration is high enough. The relative skin sensitising potency has been estimated in a mouse Local Lymph Node Assay (LLNA) by calculating the concentration of the chemical required to cause a stimulation index of 3 (EC3 value). Multiple

tests were performed in two laboratories to evaluate the intra- and inter-laboratory variation. The EC3 value for p-phenylenediamine varied between 0.06% and 0.20%.

Ref.: 126

There is plenty of evidence confirming that p-phenylenediamine is a strong clinical contact allergen in humans. The range of sensitisation responses is dependent on the vehicles, exposure condition and challenge concentration.

Standard patch tests in more than 36.000 eczema patients in Germany showed a sensitisation rate of PPD contact allergy of 4.8% after standardisation for age and sex. PPD was the 5th most frequently positive standard allergen after nickel, fragrance mix, balsam of Peru and thimerosal. There was considerable regional difference in the sensitisation rates when different regions of Germany were compared.

Ref.: 112

The North American Contact Dermatitis Group reported in 1999 that in spite of its potential allergenicity, the PPD SPIN value (Significance Prevalence index Number), which corresponds to the quantitative measure of the relative clinical importance of contact allergens in the population, remained relatively stable through the periods 1984-1985 (191), 1992-1994 (197), and 1994-1996 (185). In the same time, the SPIN rank for PPD declined from 3 to 9 to 10, respectively, among the standard contact allergens. However, one should take into consideration that PPD sensitivity may be difficult to explain in some cases because PPD may cross react to the so-called para group of compounds, which contains chemicals with a similar structure used in rubber manufacturing, local anaesthetics, azo dyes used in textiles and certain drugs like sulfa-antimicrobials.

Ref.: 71

In recent years several publications have reported series of cases of severe blistering dermatitis in patients who had used PPD containing skin paints (temporary tattoos). This “epidemic” of PPD contact allergies seen in young people who follow the trendy fashion of getting semi-permanent tattoos show how a new and different type of exposure to a potent contact allergen (skin paints with PPD containing henna) may lead to severe allergic contact dermatitis following the usual exposure through use of permanent hair dyes.

Ref.: 49, 66, 119, 125

There is marked inter-individual sensitivity to the PPD molecule on patch testing, with regard to both the exposure time and the concentration required. Experiments using PPD allergic patients showed that 6 of 16 reacted to 1% PPD after only 15 min exposure.

Ref.: 78

Further, recent reports have described serious contact anaphylaxis due to PPD in hair dyes. These immediate hypersensitivity reactions appear to be rare.

Ref.: 101

The prevalence of positive patch tests to PPD in consecutive eczema patients tested has remained rather stable over the last 30 years in spite of an increased usage of hair dyes. This finding may be associated with higher dye purity, improved formulation technology, clear use instructions and warnings on package labels. However, hair dye allergic contact dermatitis is not infrequent and often leads to very severe bouts of oozing scalp dermatitis requiring specialist care and often treatment with systemic corticosteroids.

Additional reference
Human data

Serious adverse skin reactions to permanent hair dyes have been reported for 8 children below 16 in Denmark. 6 of them had a previous reaction to black tattoos.

Ref.: F

From submission, December 2005
Animal data
Local lymph node assay (LLNA)

Guideline:	OECD 429
Species:	CBA/J mice
Group:	25 animals, 5 per group (female)
Substance:	p-phenylenediamine (PPD) (supplier code OR10432)
Batch:	99E483
Purity:	100% (analytical documentation not supplied)
Concentration:	0.05%, 0.25%, 1.25% (w/v)
Dose:	25 µl
Vehicle:	acetone:olive oil (AOO) - 4:4
Negative control:	vehicle
Positive control:	hexyl cinnamic aldehyde (HCA) batch no. 01016AQ at 50% (v/v)
GLP:	in compliance

On Days 0, 1 and 2, the animals were treated with the test item formulation, positive control or vehicle on the dorsal surface of each ear. On Day 5, mice were injected intravenously with 21.4 µCi of ^{3}H-methyl-thymidine. 5 hours later, the mice were sacrificed and the draining auricular lymph nodes were excised. A single cell suspension was prepared for each animal. Cells were precipitated with 5% trichloro-acetic acid. Incorporation of ^{3}H-methyl-thymidine was measured by liquid scintillation counting. The mean values obtained for each group were used to calculate stimulation indices (SI). The EC3 value (the estimated concentration inducing a SI of 3) was derived by linear interpolation between two points on the SI axis.

Result
The test substance PPD at 0.05%, 0.25% and 1.25% resulted in mean SI of 2.6, 10.4 and 16.1 respectively. The EC3 value was 0.06%. The positive control (HCA) at 50% resulted in a mean SI of 27.8.

Conclusion
It is concluded that the test substance PPD is an extremely potent skin sensitiser in mice, confirming results from numerous previous studies.

Ref.: 2, subm. 12/2005

3.3.4. Dermal / percutaneous absorption

Taken from SCCNFP/0129/99, adopted in 2002

Percutaneous absorption *in vitro*

The experiments used Frantz static cells, and Dulbecco PBS containing antioxidant was used as receptor fluid. Female abdominal or breast skin was obtained at autopsy or from cosmetic surgery. Human hair was obtained from a female Caucasian volunteer. The integrity of skin membranes was tested by use of tritiated water prior to commencement of the study. Diffusion cells with high rates of water permeability or anomalously high values for PPD permeation were eliminated from the study. The percutaneous absorption of ring ^{14}C-labeled PPD to human skin over 48 hours was evaluated on 5 different dosing conditions (including a 30-minute post application aqueous rinse of the skin to mimic "in-use" conditions). The dosing conditions were:

a) 100 mg/cm^2 of 1.3% PPD and other dyes in the presence of developer, but the absence of hair
b) 100 mg/cm^2 of 1.3% PPD and other dyes in the presence of developer and hair
c) 100 mg/cm^2 of 2.7% PPD, but no other dyes, developer or hair
d) 20 mg/cm^2 of 2.7% PPD, but no other dyes, developer or hair
e) 100 mg/cm^2 of 1.3% PPD, but no other dyes, developer or hair

30 cells were included in study A and 15 in the four remaining studies. 5 mg/cm^2 of hair was placed on the skin surface before addition of the formulation in the second dosing condition (B) described above.

Results

The skin penetration was between 0.1% and 0.2% of the applied dose. This corresponded to a cumulative mass absorbed of about 1.9-2.4 µg/cm^2 for the complete dye formulations. The amount of radioactive material found in the skin itself ranged from 0.04-0.5% or 0.65-6.72 µg/cm^2. For all formulations, the maximum cumulative absorption of PPD occurred 4 hours post application. This was followed by a slowing of the permeation caused by the removal PPD by the 30-minute aqueous rinse. Permeation was concentration and dose related. The presence of hair on the surface did not significantly affect the permeation process. A greater amount of PPD was found on or in the skin (but not in the receptor fluid) when it was applied in the presence of developer and other dyes and in the presence of hair. The study also included mass balance calculations showing a recovery rate between 83.6% and 104%. In conclusion this study has given results very close to the permeation levels found *in vivo* in humans (Wolfram and Maibach study, 1985). These percentage values corresponded to cumulative mass absorbed of about 1.9-2.4 µg/cm^2 for the *in vitro* studies and 4.5 µg/cm^2 for the *in vivo* studies.

Ref.: 2

Percutaneous absorption *in vivo*

This study is published in a peer reviewed journal. A commercially available hair dye product (Nice'n Easy Blue Black) containing 2.7% of PPD was enriched with ring ^{14}C-labelled PPD. Scalp penetration under condition of hair dye usage was evaluated for both rhesus monkey and man. The study included 5 human volunteers and 3 rhesus monkeys. The 2 species showed a remarkable similar pattern of dye penetration. Their amount absorbed was quantified on the basis of the percentage of radioactivity excreted in the urine following the application of known amount of labelled compound. Urine collection was continued as long radioactivity was recoverable in human and in the monkeys for 7 days. The total dose excreted in the urine in

humans was 0.190±0.06%, and 0.182±0.06% in monkeys. Using these data, Kalopissis calculated the total exposure based on cumulative mass absorbed per scalp to be 3129 μg equivalent to 4.47 μg/cm^2, based on an estimated scalp area of 700 cm^2.

Ref.: 58, 129

An analytical method was developed to determine PPD derivatives in urine samples collected from women after hair dyeing with commercial formulation. Five volunteers participated in the experiments. Metabolites of PPD were hydrolysed and measured using gas-chromatography mass-spectrometry. In the study the excretion of metabolites of PPD was followed for 2 days after the dye had been applied. The dose related excretion for PPD as measured by this method was comparable to that found by other authors who made use of tracer labelled material.

Ref.: 43

A further study in humans was submitted using radiolabelled PPD in an oxidative hair dye formulation. The study is discussed under 3.3.9. (Reference 4 and 5).

3.3.5. Repeated dose toxicity

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

Taken from SCCNFP/0129/99, adopted in 2002

A 14-day study was conducted according to OECD Guideline n° 408 (1981) in five groups of 20 (10 males and 10 females) rats from the Crl: CD (SD) BR strain (VAF plus) receiving PPD by gavage.
The animals received daily the test article dissolved in deionised, boiled water at dose levels of 5, 10, 20 and 40 mg/kg/day (free base) ; the animals of the control group were treated with the vehicle alone. All doses were given under the same volume 10 ml/kg bw.

- No treatment-related effects were noted on deaths, clinical observations, body weight growth, food intake, haematological parameters, macroscopic observations at necropsy.
- Treatment related blood chemistry changes resulted in both sexes at dose level of 5 mg/kg/day or greater (increased lactate dehydrogenase and creatine phosphokinase levels).
- Mean absolute and relative liver weights raised in males given 40 mg/kg bw/day while mean relative thyroid weights raised in females given 10 mg/kg bw/day and greater.
- Histopathological treatment findings were restricted to minimal myodegeneration noted in the skeletal muscle of 3 females given 40 mg/kg bw/day.

Under the experimental conditions adopted the NOAEL was < 5 mg/kg bw/day.

Ref.: 120

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

Taken from SCCNFP/0129/99, adopted in 2002

Oral toxicity

A 13-week study was conducted in 150 Crl:CD(SD)BR rats (5 groups, 15 animals per sex) according to the OECD guideline N° 408 (1981). PPD was administered by gavage at corresponding dose levels of 2, 4, 8 and 16 mg/kg/day while the control group received the vehicle only (deionised boiled water). All doses were administered under a same 10 ml/kg bw volume. The animals were examined twice daily for mortality/viability and once daily for clinical signs. Food consumption and body weight were recorded weekly during pre-test and treatment period and before necropsy. Ophthalmoscopic examination was performed at pre-test and at week 13 (control and high-dose animals). Weeks 4 and 12/13, blood samples were collected for haematology and clinical biochemistry from all animals; urine samples were collected for urinalysis. After 13 weeks, all animals were weighed and necropsied and descriptions of all macroscopic abnormalities were recorded. The major tissues and organ were collected from all animals; absolute and relative weights were recorded at necropsy for adrenals, brain, heart, kidneys, liver, ovaries, spleen, testes, thyroid, thymus and pituitary. Macroscopic and microscopic examination of a complete set of tissues from control and high dose animals were performed.

There were no mortalities or clinical signs considered related to the test article. No effect of PPD was recorded on the relative food consumption in any group. Body weights and body weight gain were unaffected by the treatment. No ophthalmologic findings related to the product tested were noted. Concerning haematology, blood chemistry or urinalysis parameters, no changes were considered to be related to test article administration.
The mean absolute and body-weight-related liver weights were significantly increased for males given 8 mg/kg/day and 16 mg/kg/day. At the same dose levels, absolute and body weight-related kidney weights were increased for females. However, no associated histopathological changes were noted. No treatment macroscopic or microscopic findings were recorded. Histopathological examination restricted significant finding to minimal myodegeneration on skeletal muscle on 1 male and 1 female of the high dose group (16 mg/kg bw).
Based on these results, the NOAEL of PPD was established at 4 mg/kg/day.

Ref.: 121

A 12-week oral toxicity study was conducted in F344 rats (10 – 11 rats per group) with PPD administered in the diet at concentrations of 0.05 %, 0.1 %, 0.2 % and 0.4 % (or approximately 25, 50, 100 and 200 mg/kg/day respectively). Mortalities were observed at the theoretical dose of 200 mg/kg bw in 9 male rats and in 1 female rat. At the same dose level, a 50 % reduction in body weight in both sexes as compared to controls, as well as increased relative liver and kidney weights were noted. A trend toward these above observations was noted at the theoretical dose 100 mg/kg bw.
Based on these results, the NOAEL was obtained with dosing 50 mg/kg bw.

Ref.: 53

Another 13-week neurotoxicity study has been performed via gavage in young adult F344 strain rats to evaluate the potential neurotoxicity of PPD. Male and female ccl:CD BR Rats (10 rats/sex/group) were administered PPD at doses of 4, 8 and 16 mg/kg bw/day while the control group received the vehicle only (sterile water for injection). All doses were given under a same dosage volume of 10 ml/kg bw.
The animals were examined daily for mortality and clinical signs. Food consumption and body weights were recorded at least weekly. Ophthalmological examinations were performed before and at the end of dosing. Neurotoxicity evaluations were conducted before and after 4, 8 and 13weeks of dosing according to a test battery consisting in motor activity and functional battery assessments.
There were no mortalities or clinical signs considered related to the test article. Food intake and body weight gain of treated group were similar to the controls. At 16 mg/kg bw/d increased incidence of wet chin in both sexes and in wet inguen and/or wet perineum in females was observed. Neuropathology evaluations did not reveal abnormalities within the nervous system or skeletal muscle. There was no effect of the test substance on ocular tissue.

The effects observed at 16 mg/kg bw/d are judged as pharmacological responses. Therefore, the NOEL was 8 mg/kg for both sexes, the NOAEL was 16 mg/kg bw.

Ref.: 46

Dermal toxicity

A 90 day study has been carried out in the rabbit with the compound administered dermally twice weekly. Four hair-dye formulation containing 1, 2, 3 or 4% of para-phenylenediamine and other hair-dye constituents were mixed with an equal volume of 6% hydrogen peroxide. A dose of 1 ml/kg of this mixture was applied for 1 hour without occlusion to three application sites on six animals of each sex. The application sites were abraded prior to the first dose each week. No dose-related changes were observed on weight gain, clinical chemistry, haematology, urinalysis or on examination of the tissues at necropsy.

Ref.: 20

3.3.5.3. Chronic (> 12 months) toxicity

Taken from SCCNFP/0129/99, adopted in 2002

A 80-week study was performed in F344 rat in order to assess simultaneously the long term toxicity and potential carcinogenicity of PPD when administered daily in diet at concentrates of 0.05% or 0.1%, corresponding to approximately 25 or 50 mg/kg bw/day.
In absence of the full report of the study and basing on the information reported in Submission III, at concentrations of 0.05% and 0.1%, mean absolute spleen weight in females was lower than that of controls.
No other adverse effect related to a potential toxicity of PPD was observed at the dose level used.

Ref.: 53

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1. Mutagenicity / Genotoxicity *in vitro*

p-Phenylenediamine alone

Taken from Submission III, COLIPA, June 2000

Gene mutation test in mammalian cells (mouse lymphoma assay, $tk^{+/-}$ locus)

p-Phenylenediamine was reported to be positive in the *in vitro* L5178Y mouse lymphoma assay with and without metabolic activation. Without S9, several trials demonstrated positive results using concentrations ranging from 2.1 - 6.5 µg/ml. A concentration-related increase in mutagenicity was observed and a three-fold increase was recorded at 6.5 µg/ml. With S9 positive results were obtained when tested at concentrations ranging from 7- 300 µg/ml. A two-fold increase in mutant colonies was observed at 250 µg/ml.

Ref.: 79

Taken from SCCNFP/0129/99, adopted in 2002. The text was modified.

Bacterial gene mutation test

p-Phenylenediamine has been tested for mutagenicity in *Salmonella typhimurium* strains TA98 and TA1538 in the presence of S-9. The assay was performed with the plate incorporation procedure using duplicate plates. Either water or DMSO was used as solvent.

Result:
Aqueous or fresh DMSO solutions of p-phenylenediamine did not induce an increase in the number of revertant colonies.

Conclusion:
Under the experimental conditions used p-phenylenediamine is not genotoxic (mutagenic) in this gene mutation tests in bacteria.

Ref.: 25

Comments
The paper indicates that the time between the preparation of the p-phenylenediamine solution and its use in the Ames test may influence the mutagenicity observed; DMSO used as a solvent may also influence the mutagenic response of the TA98 and TA1538 in the presence of a metabolic activation system.
The absence of information about the chemical purity of the sample of p-phenylenediamine makes the results less relevant.

Bacterial gene mutation test

Commercial samples of analytical grade p-phenylenediamine, a purified p-phenylenediamine sample (from the commercial sample), and a commercial sample of resorcinol have been tested

for mutagenicity on TA98 strain of *Salmonella typhimurium* in the presence and absence of S-9 mix obtained from rat-livers treated with Aroclor. The doses tested ranged from 0 – 2000 µg/plate. Three replicate plates were evaluated.

Results
A 100-fold increase compared to the control value was observed for the commercial samples of p-phenylenediamine whereas no increase in revertant colonies was found for purified p-phenylenediamine and for commercial resorcinol.

Conclusion
Under the experimental conditions used a commercial sample of analytical grade p-phenylenediamine is genotoxic (mutagenic) in this gene mutation tests in bacteria.

Ref.: 30

Bacterial gene mutation test

p-Phenylenediamine was tested in the gene mutation test in bacteria. Next to TA98 and TA 100 the nitroreductase-deficient strains TA98NR and TA100NR of *Salmonella typhimurium* in the presence and absence of S-9 mix using the pre-incubation procedure were exposed. Concentrations of 1 – 3000 µg/plate were tested. S-9 mix obtained from rat-livers treated with Aroclor was used.

Results
Exposure to p-phenylenediamine did not result in an increase in revertants in strains TA98 and TA100. In TA98NR an increase in the number of revertants was seen in the presence of S9; in TA100RN in the absence of S9.

Conclusion:
Under the experimental conditions used p-phenylenediamine is genotoxic (mutagenic) in this gene mutation tests in bacteria.

Ref.: 28

Chromosome aberration test

p-Phenylenediamine was tested in an chromosome aberration using CHO-K1 cells. These cells were exposed in the absence of S9 to p-phenylenediamine for 2 h followed by a 20 h culture in fresh medium, the last 3 h in combination with colchicine. The test concentrations ranged from 15 – 87 µg/ml.

Results
Exposure of CHO-K1 cells to p-phenylenediamine resulted in a slight increase in the percentage of aberrant cells compared to concurrent controls.

Conclusion:
Under the experimental conditions used p-phenylenediamine is genotoxic (clastogenic) in this chromosomal aberration test.

Ref.: 28

Submitted 2005

Bacterial gene mutation assay

Guideline:	OECD 471	
Species/strain:	*Salmonella typhimurium* TA98, TA100, TA102, TA1535, and TA1537.	
Replicates:	3 replicates in 2 individual experiments both without and with S9.	
Test substance:	p-phenylenediamine HCl	
Solvent:	degassed purified water	
Batch:	000 D 002	
Purity:	> 99%	
Concentrations:	experiment 1:	1.6 – 5000 µg/plate both without and with S9
	experiment 2:	156.3 - 5000 µg/plate both without and with S9
Treatment:	experiment 1:	direct plate incorporation (72 h treatment)
	experiment 2:	pre-incubation method (60 minutes treatment)
	additional direct plate incorporation method for TA98 with S9	
GLP:	in compliance	

p-Phenylenediamine was investigated for the induction of gene mutations in *Salmonella typhimurium* (Ames test) using the direct plate incorporation method (experiment 1 with 72 h exposure) or the pre-incubation method (experiment 2 with pre-incubation of 60 minutes). Liver post-mitochondrial S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Doses were chosen after a toxicity range-finder experiment with strain TA100 in the absence and presence of S9 using doses up to 5000 µg/plate. Since p-phenylenediamine is an oxidative compound it is was stored and diluted under nitrogen atmosphere and flushed with nitrogen before and after the dilution steps. Toxicity was reported as a thinning of the bacterial lawn.
Negative and positive controls were in accordance with the OECD guideline.

Results
In the toxicity range-finder experiment with TA100 no evidence of toxicity was observed. In experiment 1 evidence of toxicity as a slight thinning of the bacterial background lawn was only found in TA102 after the highest dose both without and with S9. In experiment 2, toxicity was found in the plate incorporation treatments of TA98 with S9 at the highest dose. Using the pre-incubation method toxicity was found for TA102 at 2500 and 5000 µg/plate with S9 and at 5000 µg/plate without S9.
In experiment 1, statistically significant increases were found in TA100 without S9 only. Since a dose response relationship was absent and the increase was not reproducible in experiment 2, it was concluded that the increase was not biologically relevant. In both experiments independent of the treatment method but a positive result was found in strain TA98 in the presence of S9 only. The increase was dose related in experiment 1 and more or less plateaued in experiment 2.
A p-phenylenediamine treatment related increase in the number of revertant colonies as compared to concurrent vehicle controls was not observed in any of the other *Salmonella* strains

Conclusion
Positive results were found in the presence of S9 metabolic activation with strain TA98. Therefore, under the experimental conditions used p-phenylenediamine is genotoxic (mutagenic) in this gene mutation tests in bacteria.

Ref.: 3, subm. 12/2005

In vitro Gene Mutation Assay (*hprt* locus)

Guideline:	OECD 476	
Species/strain:	mouse lymphoma L5178Y cells	
Replicates:	duplicates in two independent experiments	
Test substance:	p-Phenylenediamine HCl	
Solvent:	purified water	
Batch:	000 D002	
Purity:	99.3 ± 0.5%	
Concentrations:	experiment 1:	2.5 – 80 µg/ml without S9
		25 – 900 µg/ml with S9
	experiment 2:	2.5 – 60 µg/ml without S9
		25 – 1000 µg/ml with S9
Treatment	3 h treatment with a 7 days expression period followed by a 6-TG selection period of 10-12 days	
GLP:	in compliance	

p-Phenylenediamine was assayed for gene mutations at the *hprt* locus in mouse lymphoma cells using a fluctuation protocol both in the absence and presence of S9 metabolic activation. Test concentrations were based on the level of toxicity in a cytotoxicity range finding experiment. Cells were treated for 3 h followed by an expression period of 7 days to fix the DNA damage into a stable *hprt* mutation and a selection period for 6-TG mutations of 12 days. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Toxicity was measured as percentage relative survival of the treated cultures relative to the survival of the solvent control cultures. Negative and positive controls were in accordance with the OECD guideline.

Results

No significant changes in osmolarity were observed at the highest dose tested compared to the concurrent controls. A decreased pH was seen in the cytotoxicity range finding study but not in experiment 1.

In experiment 1 without S9 the highest dose tested has a relative survival of 8.41% and is therefore too toxic. The closest dose, however, has a relative survival of 30.6% which is too high. The necessary intermediate dose is lacking. In experiment 1 with S9 and in the second experiment in the absence and presence of S9 the appropriate level of toxicity (10-20% survival after the highest dose) was reached pointing to sufficient exposure of the cells.

In both experiments a dose dependent and biologically relevant increase in the mutation frequency at the *hprt* locus of mouse lymphoma cells was not observed. The only statistically significant increase in the mutation frequency (experiment 2 with S9 intermediate dose of 100 µg/ml) was not reproducible and therefore considered not biologically relevant.

Conclusion

Under the experimental conditions used p-phenylenediamine treatment did not result in an increase of the mutation frequency at the *hprt* locus of mouse lymphoma cells and, consequently, is not mutagenic in this *in vitro* gene mutation assay with mammalian cells.

Ref.: 4, subm. 12/2005

***In vitro* micronucleus test**

Guideline:	OECD draft guideline 487, in accordance with recommendations of IWTG workshop and accepted scientific/regulatory principles described in current guidelines for clastogenicity testing *in vitro*.	
Cells:	human lymphocytes of 2 healthy, non-smoking, female volunteers (under 35 years of age)	
Replicates:	duplicate cultures in 2 independent experiments with and without S9	
Test substance:	p-Phenylenediamine HCl	
Solvent:	purified water	
Batch:	000 D 002	
Purity:	99.3 %	
Concentrations:	experiment 1:	3.73, 30 and 80 µg/ml without S9 500, 900 and 1600 µg/ml with S9
	experiment 2:	50, 100 and 125 µg/ml without S9 400, 1400 and 2000 µg/ml with S9
Treatment	experiment 1:	24 h PHA, 20 h treatment and 28 h recovery without S9 24 h PHA, 3 h treatment and 45 h recovery with S9
	experiment 2:	48 h PHA, 20 h treatment and 28 h recovery without S9 48 h PHA, 3 h treatment and 45 h recovery with S9
GLP:	In compliance	
Comment:	The OECD draft guideline 487 does not suggest a protocol with a 45-h recovery when the treatment was performed 48 h after mitogen stimulation	

p-Phenylenediamine has been investigated in 2 independent experiments in the absence and presence of metabolic activation for the induction of micronuclei in cultured human lymphocytes. Since p-phenylenediamine is an oxidative compound it is was stored and diluted under nitrogen atmosphere.
Treatment periods were 24 h without S9 and 3 h with S9. Harvest times were 72 hours (experiment 1) or 96 hours (experiments 2) after the beginning of culture. Approximately the final 28 h of incubation was in the presence of cytochalasin B (at a final concentration of 6 µg/ml). Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. The range of test concentration was based on the result of a range finding toxicity study. Toxicity was determined by measuring the reduction in replication index (RI).
In every separate experiment various dilutions of p-phenylenediamine were tested. However, only 3 doses were analyzed. The top dose for analysis was to be the one with at least 50 - 60% reduction in cytotoxicity. The lower doses were chosen such that a range from maximum to little or none cytotoxicity is covered. Micronucleus preparations were stained with Giemsa and examined microscopically for RI and micronuclei. Negative and positive controls were in accordance with the OECD draft guideline.

Results
Measurements on post-treatment media in the absence or presence of S9 indicated that p-Phenylenediamine had no marked effect on osmolarity or pH as compared to concurrent vehicle controls.
In experiment 1 without S9 metabolic activation p-phenylenediamine did not induce an increase in the number of micronuclei compared to the concurrent untreated controls. One of the two cultures of the mid dose showed an increase in micronucleated bi-nucleated cells compared to the historical control value. As all other treated cultures of experiment 1 without S9 fell within this range this increase was not considered biologically relevant.

In experiment 1 with S9 as well as in experiment 2 both without and with S9 a more or less dose dependent increase in micronucleated bi-nucleated cells was observed.

Conclusion
Under the experimental conditions used p-phenylenediamine induced micronuclei after 24 h PHA stimulation in the presence of S9 metabolic activation and after 48 h PHA stimulation in both the absence and presence of S9 metabolic activation. Consequently, p-phenylenediamine is genotoxic (clastogenic and/or aneugenic) in human lymphocytes *in vitro*.

Ref.: 5, subm. 12/2005

Tests with two metabolites of p-phenylenediamine

Bacterial gene mutation assay

Guideline:	/
Species/strain:	*Salmonella typhimurium* TA98, TA100, TA102, TA1535, and TA1537.
Replicates:	3 replicates in 2 individual experiments without S9.
Test substance:	N-monacetyl-para-phenylenediamine (MAPPD) and N,N'-diacetyl-para-phenylenediamine (DAPPD)
Solvent:	MAPPD: purified water; DAPPD: DMSO
Batch:	/
Purity:	> 95%
Concentrations:	experiment 1: 1.6 – 5000 µg/plate without S9 experiment 2: 156.3 - 5000 µg/plate without S9
Treatment:	direct plate incorporation (72 h treatment)
GLP:	/

N-monacetyl-para-phenylenediamine (MAPPD) and N,N'-diacetyl-para-phenylenediamine (DAPPD) were investigated for the induction of gene mutations in *Salmonella typhimurium* (Ames test) using the direct plate incorporation method (with 72 h exposure) Toxicity was reported as a thinning of the bacterial lawn.
Negative and positive controls were in accordance with the OECD guideline.

Results
In both experiments treatment of Salmonella with MAPPD or DAPPD did not result in toxicity or precipitation. In none of the tester strains biologically relevant increases in revertant colonies were observed after treatment with MAPPD. Also following DAPPD treatment no noteworthy increases in revertant colonies were observed in any of the tester strains. A small increase in TA102 revertants at an intermediate DAPPD dose in one experiment was neither dose-related nor reproducible and was therefore considered not biologically relevant.

Conclusion
Under the experimental conditions used MAPPD and DAPPD were not genotoxic (mutagenic) in this gene mutation tests in bacteria.

Ref.: G

Comment: Since this gene mutation assay in bacteria was available in the form of a publication (in press), the raw data were not available. Moreover, the results from the top three doses only were reported.

Although it was unclear whether the test was performed according an OECD guideline, the batch was not reported and the GLP status is nut clear, the data obtained with p-phenylenediamine which were also reported in this paper were those from ref.: 3, subm. 12/2005. These data were obtained in a GLP study according an OECD guideline.

***In vitro* micronucleus test**

Guideline:	in accordance with recommendations of IWTG workshop	
Cells:	human lymphocytes of 2 healthy, non-smoking, female volunteers (under 35 years of age)	
Replicates:	duplicate cultures in 2 independent experiments without S9	
Test substance:	N-monacetyl-para-phenylenediamine (MAPPD) and N,N'-diacetyl-para-phenylenediamine (DAPPD)	
Solvent:	MAPPD: purified water; DAPPD: DMSO	
Batch:	/	
Purity:	> 95 %	
Concentrations:	MADDP:	769, 961 and 1502 µg/ml without S9
	DAPPD:	experiment 1: 480, 600 and 700 µg/ml without S9
		experiment 2: 384, 480 and 690 µg/ml without S9
Treatment	experiment 1:	24 h PHA, 20 h treatment and 28 h recovery without S9
	experiment 2:	48 h PHA, 20 h treatment and 28 h recovery without S9
GLP:	/	

N-monacetyl-para-phenylenediamine (MAPPD) and N,N'-diacetyl-para-phenylenediamine (DAPPD) have been investigated in 2 independent experiments in the absence of metabolic activation for the induction of micronuclei in cultured human lymphocytes.
Treatment periods were 24 h. Harvest times were 72 hours (experiment 1) or 96 hours (experiments 2) after the beginning of culture. Approximately the final 28 h of incubation was in the presence of cytochalasin B (at a final concentration of 6 µg/ml). Toxicity was determined by measuring the reduction in replication index (RI). The ranges of test concentrations were selected by evaluating the effect on the replication index. The top dose for analysis was to be the one with at least 50 - 60% reduction in cytotoxicity. Micronucleus preparations were stained with Giemsa and examined microscopically for RI and micronuclei. Negative and positive controls were in accordance with the OECD draft guideline.

Results
For MADDP the highest concentration chosen for analysis, 1502 µg/ml which is also the maximal dose (10mM), was not toxic and induced approximately 4% reduction in RI. In the first experiment the highest dose chosen for analysis of DAPPD, 700 µg/ml, was in excess of the solubility limit of the test compound in culture medium but was not toxic and induced approximately 11% reduction in RI. In the second experiment the top dose, 690 µg/ml, was still in excess of the solubility limit, was again not toxic, and induced approximately 91% reduction in RI.
Treatment with MADDP or DADDP did not induce a biologically relevant increase in the number of micronucleated lymphocytes compared to the concurrent untreated controls.

Conclusion
Under the experimental conditions used MADDP or DADDP is not genotoxic (clastogenic and/or aneugenic) in human lymphocytes *in vitro*.

Ref.: G

Comment
Since this *in vitro* micronucleus test was available in the form of a publication (in press), the raw data were not available.
Although it was unclear whether the test was performed according an OECD guideline, the batch was not reported and the GLP status is nut clear, the data obtained with p-phenylenediamine which were also reported in this paper were those from ref.: 3, subm. 12/2005. These data were obtained in a GLP study according an OECD guideline.

p-Phenylenediamine and hydrogen peroxide

Taken from SCCNFP/0129/99, adopted in 2002. The text was modified.

Bacterial gene mutation test

An oxidation product obtained from a reaction between purified p-phenylenediamine and Resorcinol was tested in a gene mutation assay in bacteria (Ames test). This reaction product has been identified as the "green compound", an oxidised conjugation product of p-phenylenediamine and Resorcinol, distinct and different from the Bandrowski's base. *Salmonella typhimurium* strain TA98 was exposed in the presence and absence of S-9 mix obtained from rat-livers treated with Aroclor. The doses tested ranged from 0 - 2000 μg/plate. Three replicate plates were evaluated.

Results
A 5-fold increase in the number of revertants compared to the value of the control was observed.

Conclusion
Under the conditions of the tests the oxidation product of resorcinol and p-phenylenediamine is not genotoxic (mutagenic) in this gene mutation tests in bacteria.

Ref.: 30

Bacterial gene mutation test

A compound formed as a reaction product between Resorcinol (pure commercial product from Hoechst) and p-phenylenediamine in the presence of an oxidising agent such as hydrogen peroxide, has been tested for mutagenicity in the Ames test, in the absence and presence of an activation system provided by Aroclor treated rat liver microsomes. Five *Salmonella typhimurium* strains, TA98, TA100, TA 1535, TA1537, TA1538, were used. The doses ranged from 5 – 1000 μg/plate in two independent experiments. The chemical tested was synthesised and purified by re-crystallisation, and analysed by nuclear magnetic resonance spectroscopy (NMR^1H).

Results
Resorcinol as well as the oxidation product did not induce an increase in the number of revertants in any of the 5 strains.

Conclusion

Under the conditions of the tests the oxidation product of resorcinol and p-phenylenediamine is not genotoxic (mutagenic) in this gene mutation tests in bacteria.

Ref.: 108

Comment
As p-phenylenediamine alone, the other partner reactive chemical present in many hair dye formulations was not included in this test, no conclusions can be drawn about the possible origin of mutagenicity of p-phenylenediamine combined with other chemicals.
The authors mentioned that in their previous tests p-phenylenediamine was mutagenic in the Ames test when Aroclor induced S9 was used.

Bacterial gene mutation test

Mixtures of a technical grade sample of pure p-phenylenediamine (= 99%), Resorcinol and hydrogen peroxide or p-phenylenediamine and hydrogen peroxide at concentrations relevant to practical hair dyeing procedure were assayed in the gene mutation test with bacteria (*Salmonella typhimurium* strain TA98 in the presence of S9 mix). The mixture has been evaluated after preparation and after 30 min of incubation at 37° C.

Results
p-Phenylenediamine was mutagenic in *Salmonella typhimurium* TA98 after 30 min of incubation when combined with H_2O_2. However, the mixture with resorcinol and H_2O_2 was not mutagenic.

Conclusion
It can be concluded that *in vitro* p-phenylenediamine in a mixture with H_2O_2 is genotoxic (mutagenic) for bacteria. The positivity is probably due to the formation of Bandrowski's bases which could be demonstrated with thin layer chromatography.
The mixture of p-phenylenediamine with resorcinol and H_2O_2 is not genotoxic (mutagenic) in bacterial.

Ref.: 16

Comment
In the same paper the authors reported that pre-incubation of 5 oxidative mixtures of p-phenylenediamine with various couplers at 37°C for 0-7 h resulted in mutagenicity in the gene mutation test in bacteria (TA98 with S9 mix) ranging from very strong effects to not mutagenic.
The authors concluded that oxidative hair dye mixtures of p-phenylenediamine and non-mutagenic couplers are not mutagenic if tested under normal conditions of use.

In vitro gene mutation test in mammalian cells

Mixtures of a technical grade sample of pure p-phenylenediamine (= 99%), Resorcinol and hydrogen peroxide or p-phenylenediamine and hydrogen peroxide at concentrations relevant to practical hair dyeing procedure were assayed in the mammalian gene mutation system using mouse lymphoma L5178Y cells. The mixture has been evaluated after preparation and after 30 min of incubation at 37° C.

Results:
p-Phenylenediamine was not mutagenic in mouse lymphoma cells in the presence of H_2O_2 alone nor in combination with resorcinol and H_2O_2.

Conclusion
It can be concluded that *in vitro* p-phenylenediamine in a mixture with H_2O_2 is not genotoxic (mutagenic) for mammalian cells.
The mixture of p-phenylenediamine with resorcinol and H_2O_2 is also not mutagenic in mammalian cells.

Ref.: 16

In vitro chromosome aberration test

Mixtures of a technical grade sample of pure p-phenylenediamine (= 99%), Resorcinol and hydrogen peroxide or p-phenylenediamine and hydrogen peroxide at concentrations relevant to practical hair dyeing procedure were assayed in the chromosomal aberration test with human lymphocytes. The mixture has been evaluated after preparation and after 30 min of incubation at 37° C.

Results:
Treatment of human lymphocytes with p-phenylenediamine resulted in an increase of cells with chromosomal aberrations when p-phenylenediamine was mixed with H_2O_2**.** The combination with resorcinol and H_2O_2 was not clastogenic.

Conclusion
It can be concluded that *in vitro* p-phenylenediamine in a mixture with H_2O_2 is genotoxic (clastogenic) for human lymphocytes.
The mixture of p-phenylenediamine with resorcinol and H_2O_2 is not genotoxic (clastogenic) in human lymphocytes.

Ref.: 16

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

p-Phenylenediamine alone

Taken from Submission III, COLIPA, June 2000

***In vivo* micronucleus assay**
In an in vivo oral micronucleus assay in the rat, no clastogenic activity of p-phenylenediamine was seen at 300 mg/kg bw.

Ref.: 52

Single cell gel electrophoresis assay (Comet assay)
p-Phenylenediamine was tested in the single cell gel electrophoresis (CSG or Comet) assay. A single oral (gavage) dose of 75 mg/kg (the maximum tolerated dose) was administered to four groups of male mice (4 animals per group). Animals were killed at 0, 3, 8, or 24 h after treatment and samples from eight organs – stomach, colon, liver, kidney, urinary bladder, lung, brain, and bone marrow – were taken. Slides were prepared for evaluation by the alkaline SCG assay; 50 nuclei per organ per moue were examined.
p-Phenylenediamine was negative for induction of DNA damage under the conditions of this test.

Ref.: 102

Taken from SCCNFP/0129/99, adopted in 2002. The text was modified.

***In vivo* Micronucleus Assay of p-Phenylenediamine**

Guideline:	\
Species/strain:	CD-1 mice
Group size:	5 male mice
Test substance:	p-Phenylenediamine dihydrochloride
Batch no:	\
Purity:	\
Dose level:	25, 50 and 100 mg/kg bw
Route:	ip injection
Vehicle:	Sterile distilled water
Sacrifice times:	24, 48 and 72 h for all concentrations
GLP:	Not in compliance

Rationale for the doses: 200 mg/kg killed 3/5 animals. 100 mg/kg was the highest dose tested. Positive and negative controls were included.

Results
The ratio PCE/NCE was slightly reduced at 24h (100 mg/kg), at 48 h (25 mg/kg) and at 72 h (50 and 100 mg/kg). p-Phenylenediamine did not induce a significant and biologically relevant dose or sampling time related increase in cells with micronuclei. In the mid dose at 24 and 48 a slight

increase in cells with micronuclei seems to occur which, however, decreased again at the highest dose tested and was not observed at 72 h.

Conclusion
Under the experimental conditions used, p-phenylenediamine is not genotoxic (clastogenic and/or aneugenic) in this bone marrow of mice.

Ref.: 113

Submitted in 2005

Bone marrow micronucleus test in rats

Guideline:	OECD 474
Species/strain:	Wistar rats
Group size:	5 rats per sex
Test substance:	p-Phenylenediamine
Batch no:	1365
Purity:	99.8%
Dose level:	25, 50 and 100 mg/kg bw
Route:	orally
Vehicle:	deionised water
Sacrifice times:	24 h for all concentrations, 48 h for the vehicle control and the highest dose.
GLP:	In compliance

Radioactive-labelled test substance:

Test substance:	p-[U14C]Phenylenediamine dihydrochloride
Batch:	CFQ14096 Batch 1
Radiochemical purity:	97.7%
Specific radioactivity:	2.11 GBq/mmol or 57mCi/mmol

p-Phenylenediamine has been investigated for the induction of micronuclei in bone marrow cells of rats. Since p-phenylenediamine is an oxidative compound it is degassed by sonication for at least 15 minutes and then saturated with nitrogen gas and kept under nitrogen atmosphere for 15 minutes prior to dosing.
Test concentrations were based on a preliminary study on acute toxicity. As in a parallel GLP study treatment with 150 mg/kg bw after oral (gavage) application resulted in the death of 1 out of 4 rats the starting dose for this pre-experiment for toxicity was 100 mg/kg bw.
To test the concentration of p-phenylenediamine in blood serum 3 rats per sex and sampling time (0.5 and 2 h post treatment) were treated with 100 mg/kg bw of radio-labelled p-phenylenediamine. Radioactivity in blood was determined by liquid scintillation counting.
In the micronucleus experiment rats were exposed to oral doses of 0, 25, 50 and 100 mg/kg bw. At various time points after administration (1, 2-4, 6 and 24 h) rats were examined for acute toxic symptoms. 24 h or 48 h (highest dose and concurrent vehicle control only) after dosing bone marrow cells were collected. Toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic and normochromatic erythrocytes (PCE/NCE) per 2000 erythrocytes. Bone marrow preparations were stained with May-Grünwald (one slide) and acridine orange (at least one slide) and examined microscopically for the PCE/NCE ratio and micronuclei. Negative and positive controls were in accordance with the OECD draft guideline.

Results
In the pre-experiment for toxicity, all rats demonstrated a reduction of spontaneous activity the up to 24 h after treatment. Rats killed 6 h after application showed ruffled fur and those killed at 2-4 and 6 h orange coloured urine. On the basis of these data and the results of a parallel study (showing that 150 mg/kg bw p-phenylenediamine produced mortality), 100 mg/kg bw was estimated to be the maximum tolerated dose and set as high dose.
In the micronucleus study no mortality occurred. Toxic signs following exposure were identical to that of the pre-experiment for toxicity but slightly decreased in severness with the dose.
The PCE/NCE ratio was not decreased after exposure to p-phenylenediamine indicating that p-phenylenediamine had no cytotoxic properties in the bone marrow. However, the toxic signs after application indicated to systemic exposure of the bone marrow cells. Also measurement of plasma levels demonstrated significant levels of p-phenylenediamine in blood and plasma (> 20 µg eq p-phenylenediamine/g plasma) indicating that after oral administration p-phenylenediamine was rapidly absorbed in significant amounts.
After May-Grünwald staining a dose dependent increase in the number of micronucleated bone marrow cells isolated 24 h after application was seen. According to the authors, this increase was within the range of the historical controls (0.078-0.245%). However, the increase after the highest dose was exactly identical to the top level of the range of the historical controls (0.245%). An increase, again within the range of the historical controls, was also seen in bone marrow cells isolated 48 h after treatment. Acridine orange staining demonstrated an increase of micronucleated bone marrow cells at 24 h but not at 48 h. At the lowest dose the increase is even statistically significant as compared to the concurrent control. The increases are again within the historical control range.

Conclusion
Since the increases in micronucleated bone marrow cells were all within the range of the historical controls, under the experimental conditions used, p-phenylenediamine it is concluded that for the time being the increase in micronuclei in bone marrow cells of treated rats is considered not biological relevant and, consequently, p-phenylenediamine is provisionally considered not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of rats.

Ref.: 6, subm. 12/2005

Remark
To come to a definitive conclusion on the results of p-phenylenediamine in this micronucleus test in rats, the company has to deliver more details on the historical control data and the range of these historical control data.

In vivo Unscheduled DNA Synthesis (UDS) test in rat

Guideline:	OECD 486 and EC B39
Species/strain:	Wistar Hanlbm: WIST (SPF) rats
Group size:	3 male rats
Test substance:	p-Phenylenediamine
Batch:	1365
Purity:	99.8%
Dose level:	50 and 100 mg/kg bw
Route:	oral, once
Vehicle:	deionised water

Sacrifice times: 2 h and 16 h after dosing
GLP: In compliance

p-Phenylenediamine was investigated for the induction of unscheduled DNA synthesis (UDS) in hepatocytes of rats. Since p-phenylenediamine is an oxidative compound it is degassed by sonication for at least 15 minutes and then saturated with nitrogen gas and kept under nitrogen atmosphere for 15 minutes prior to dosing. Test concentrations were based on a number of preliminary studies on acute toxicity. The highest dose selected was the maximum tolerated dose 100 mg/kg bw.
Hepatocytes for UDS analysis were collected at 2 h and 16 h after administration of p-phenylenediamine. Ninety minutes after plating the cells were incubated for 4 h with 5 μCi/ml ^{3}H-thymidine (specific activity 20 Ci/mmol). Evaluation of autoradiography was done after 14 days exposure.
UDS was measured by determining nuclear grain count (the number of nuclear grains minus the average number of grains in 2 heavily labelled nuclear-sized areas adjacent to each nucleus) and the mean and percentage cells in repair (cell with a net grain count larger than 5). Unscheduled synthesis was determined in 50 randomly selected hepatocytes on each of 2 replicate cultures per rat.
Negative and positive controls were in accordance with the OECD guideline.

Results
In the pre-experiments for toxicity (with 100 - 500 mg/kg bw p-phenylenediamine), the rats demonstrated a reduction of spontaneous activity, ruffled fur, coloured urine, apathy, abdominal poison and occasional death. On the basis of these data 100 mg/kg bw was estimated to be the maximum tolerated dose and set as high dose.
In the UDS test mortality was not observed. In the rats killed 2 h after application orange coloured urine and in the 16 h group reduction of spontaneous activity and coloured urine was observed indicating to systemic availability of p-phenylenediamine. Viability and the number of the isolated hepatocytes was not effected by p-phenylenediamine
Both for the 2 h and the 16 time-points after treatment none of the individual groups showed an increased mean net nuclear grain count in the hepatocytes as compared to the untreated controls. Also the number of in repair never reached the necessary criterion of 10% above the percentage found for the untreated control.

Conclusion
Under the experimental conditions used p-phenylenediamine did not induce unscheduled DNA synthesis and, consequently, is not genotoxic in rats in this *in vivo* UDS test.

Ref.: 7, subm. 12/2005

Comment
In the report a separate study on the p-phenylenediamine levels in blood and plasma of p-phenylenediamine treated rats is mentioned and used in the conclusions but not incorporated in the report.

3.3.7. Carcinogenicity

p-Phenylenediamine alone

Induction of γ-glutamyl transpeptidase-positive foci in rat liver

Guideline:	/
Species/strain:	Male F344/DuCrj rats
Group size:	25 Animals, negative control group 50 animals
Test substance:	p-Phenylenediamine (PPD)
Batch:	Lot CQ, Seiko Kagaku Co. Ltd
Purity:	99.5%
Dose:	110, 330, and 1000 ppm PPD in the diet
Route:	Oral
Exposure:	6 weeks
GLP:	not in compliance

The effect of PPD on liver carcinogenesis was investigated in male F344 rats initially treated with N-nitrosodiethylamine (DEN). Two weeks after a single dose of DEN (200 mg/kg, intraperitoneally) (6 weeks old rats at the commencement of the experiment), the rats were given PPD at dietary levels of 110, 330 and 1000 ppm for 6 weeks. At week 3 following the N-nitrosodiethylamine treatment, all animals were subjected to 2/3 partial hepatectomy. Positive control: 600 ppm 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) in the diet for 6 weeks. All survivors were sacrificed under anaesthesia for examination at week 8.

Slight retardation of body weight gain was observed in rats treated with PPD at all dietary levels. Significant increases in relative liver weight were found in animals treated with 1000 ppm PPD. Remarkable growth retardation and increased liver weight were found in rats given 3'-Me-DAB. PPD did not significantly increase the level of γ-glutamyl transpeptidase-positive foci observed after DEN initiation. Increased levels were found after treatment with the positive control 3'-Me-DAB.

Ref.: 46

Topical administration, mice

Guideline:	/
Species/strain:	Female Swiss mice
Group size:	50 Mice, negative control group 93 mice, positive control group 40 mice
Test substance:	p-Phenylenediamine (PPD)
Batch:	/
Purity:	not stated
Dose:	5% and 10% PPD in acetone
Route:	Topical
Exposure:	Twice weekly until they died spontaneously or were killed when moribund
GLP:	not in compliance

Swiss mice, 7 weeks old, groups of 50 females received 5 or 10% PPD applied in 0.02 ml (0.05 or 0.1 µg; 1 cm diameter) acetone twice weekly in a shaved area of interscapular skin. 93 untreated females served as controls and 40 mice received 7,12-dimethylbenz(a)anthracene (DMBA) (0.1%) and were kept as positive controls. The mice were allowed to die spontaneously or were killed when moribund.

There was no significant decrease in the lifespan of the treated mice. The mice suffered no marked weight loss. No treatment-related epidermal hyperplasia, ulceration or dermatitis was observed.

27 of 44 low-dosed mice (61%) had altogether 32 tumours and 24 of 49 high-dosed mice (49%) had altogether 34 tumours. 39 of the negative controls (42%) had altogether 46 tumours while 38 of the positive controls (95%) had altogether 67 tumours. The incidence of tumours in the different organs of the treated mice was not statistically different from that of untreated controls.

Ref.: 115

Topical administration, rabbits

Guideline:	/
Species/strain:	Female rabbits, strain not specified
Group size:	5 Rabbits
Test substance:	p-Phenylenediamine (PPD)
Batch:	/
Purity:	not stated
Dose:	5% and 10% PPD in acetone
Route:	Topical
Exposure:	Twice weekly until termination after 85 weeks
GLP:	not in compliance

Rabbits (strain not stated), 8 weeks old, groups of 5 females received 5 or 10% PPD applied in 0.02 ml acetone twice weekly to the inside of the left ear. The experiment was terminated at week 85. 5 untreated females served as controls and 5 rabbits treated with 7,12-dimethylbenz(a)anthracene (DMBA) (0.1%) were kept as positive controls.

No abnormalities were found in the blood or urine of the rabbits. No treatment-related local changes were observed in the ears. The positive controls showed 15 proliferating papillomas in the 5 rabbits. Tumours were not found in other organs in any of the groups.

Ref.: 115

Oral administration, mice

Guideline:	/
Species/strain:	B6C3F1 mice
Group size:	50 animals per sex and dose, control 20 male and 20 female
Test substance:	p-Phenylenediamine dihydrochloride (PPD)
Batch:	/
Purity:	not stated
Dose:	625 and 1250 ppm in the diet

Route: Oral
Exposure: 103 weeks
GLP: in compliance

US National Toxicology Program carried out the study.

B6C3F1 mice, groups of 50 males and 50 females (6 weeks old), were exposed to 625 or 1250 ppm PPD dihydrochloride in the diet for 103 weeks. After the 103 week period of compound administration, there were additional observation periods of 1 week before the remaining animals were sacrificed. Twenty animals of each sex were used as controls.

There were no significant positive association between the concentration of PPD administered and mortality of either sex. The mean bodyweights among the dosed female mice were slightly depressed in relation to their respective controls, indicating that the concentrations of PPD dihydrochloride administered to these animals in this bioassay may have approximated the maximum tolerated concentrations. Adequate numbers of animals in all groups survived sufficiently long to be at risk from late-developing tumours.

None of the statistical tests for any site in mice of either sex, including time to leukaemia or malignant lymphoma analysis in female mice, indicated a significant positive association between compound administration and tumour incidence. It was concluded that under the condition of this bioassay, there were no convincing evidence that dietary administration of PPD was carcinogenic in B6C3F1 mice.

Ref.: 86

Transplacental carcinogenicity

Guideline: /
Species/strain: Pregnant NMRI mice
Group size: 22 Animals, control 20 male and 20 female
Test substance: p-Phenylenediamine dihydrochloride (PPD)
Batch: /
Purity: not stated
Dose: 30 mg/kg by gavage
Route: Oral
Exposure: 10 days
GLP: not in compliance

Pregnant NMRI mice (a group of 22 animals) were administered 30 mg/kg PPD in soy bean oil by gavage once a day from pregnancy day 10 through day 19 (a total of 10 administrations). A positive control group was administered urethane (300 mg/kg) and vehicle administered (10 ml/kg). The F1 generation numbered 95 males and 95 females in the PPD group, 110 males and 99 females in the urethane group and 77 males and 81 females in the vehicle control group.

Total observation time was 137 weeks. PPD did not affect bodyweight or survival in dams or offspring, while the offspring of urethane treated dams had both lower survival rates and lower bodyweights compared to vehicle controls.

Tumours occurred 31.2% of the PPD-treated animals as compared to 30.5% in vehicle control animals and 70.9% in the urethane-treated animals. The most commonly observed tumours were lymphomas and alveolar adenomas in all groups. When the incidence of alveolar adenomas was calculated in F_1 females, a slight statistically significant increase was observed in PPD-treated animals compared to the vehicle controls (p=0.04). No increase in overall tumour incidence occurred in PPD treated dams or in their offspring.

Ref.: 50

Oral administration, rats

Guideline:	/
Species/strain:	F344 rats
Group size:	50 Animals per sex and dose, control 20 male and 20 female
Test substance:	p-Phenylenediamine dihydrochloride (PPD)
Batch:	/
Purity:	not stated
Dose:	625 and 1250 ppm in the diet
Route:	Oral
Exposure:	103 weeks
GLP:	in compliance

US National Toxicology Program carried out the study.

Fischer 344 rats, groups of 50 males and 50 females (6 weeks old), were exposed to 625 or 1250 ppm PPD dihydrochloride for 103 weeks. After the 103 week period of compound administration, there were additional observation periods of 2 weeks before the remaining animals were sacrificed. Twenty animals of each sex were used as controls.

There were no significant positive association between the concentration of PPD administered and mortality of either sex. Slight dose-related mean body- weight depression was observed in female rats and the mean bodyweights among high dose male rats were slightly depressed in relation to their respective controls, indicating that the concentrations of PPD dihydrochloride administered to these animals in this bioassay may have approximated the maximum tolerated concentrations. Adequate numbers of animals in all groups survived sufficiently long to be at risk from late-developing tumours.

None of the statistical tests for any site in rats of either sex indicated a significant positive association between compound administration and tumour incidence. It was concluded that under the condition of this bioassay, there were no convincing evidence that dietary administration of PPD was carcinogenic in Fischer 344 rats.

Ref.: 86

Guideline:	/
Species/strain:	F344 rats
Group size:	35 – 42 Animals per sex and dose, control 19 male and 21 female
Test substance:	p-Phenylenediamine dihydrochloride (PPD)
Batch:	/
Purity:	not stated

Dose:	0.05% and 0.1% in the diet
Route:	Oral
Exposure:	80 weeks
GLP:	not in compliance

F344 rats, 6 weeks old of both sexes, were divided into 3 groups. The groups were fed diet containing 0 (control), 0.05% (500 ppm), and 0.1% (1000 ppm) PPD respectively. The animals were killed after 80 weeks or when they became moribund.

There was no relation between the average body weight and the concentration of PPD in male rats, but the body weight of the female rats given 0.1% PPD was slightly less than of the controls. The survival was not affected significantly by the treatment. It is concluded that PPD was not carcinogenic to F344 rats of either sex when given orally.

Ref.: 53

Intraperitoneal injection, mice

Guideline:	/
Species/strain:	Strain A mice
Group size:	Lab A: 10 or 20 animals per sex and dose, control 54 males and 54 females Lab B: 30 males
Test substance:	p-Phenylenediamine dihydrochloride (PPD)
Batch:	/
Purity:	not stated
Dose:	Lab A: 12.5 and 25 mg/kg, Lab B: 6.4, 16 and 32 mg/kg
Route:	Intraperitoneal injection
Exposure:	3 times a week for 8 weeks
GLP:	not in compliance

PPD was studied in strain A mice in two different laboratories (A and B). The experiment started when the animals were 6-8 weeks old. The animals received intraperitoneal injections 3 times a week for 8 weeks.

Laboratory A. Groups of 10 or 20 males and 10 or 20 females received injections with 12.5 and 25 mg/kg with PPD.

10% (1/10) (0.10 tumours per mice) of the low dosed males and 11% (1/9) (0.10 tumours per mice) of low dosed females developed lung tumours. 20% (3/15) (0.20 tumours per mice) of the high dosed males and 29% (4/14) (0.36 tumours per mice; $P<0.05$) of high dosed females developed lung tumours. 13% (7/54) (0.167 tumours per male) of control males and 11% (6/54) (0.11 tumour per mice) of control females developed lung tumours.

Laboratory B. Groups of 30 males were used and the animals received injections with 6.4, 16 or 32 mg/kg PPD.

The percent survivors with tumours were 13% (3/23) (0.13 tumours per mice), 27% (7/26) (0.31 tumours per mice), and 30% (7/23) (0.30 tumours per mice) among the low, medium and high dosed animals respectively. Among the control males 33% (8/24) (0.42 tumours per mice) developed tumours.

With an exception of female mice in Laboratory A, all experiments were negative.

Ref.: 73

Neonatal carcinogenesis

Guideline:	/
Species/strain:	NMRI mice
Group size:	51 Males and 55 females, control 49 males and 43 females, positive control 42 males and 27 females
Test substance:	p-Phenylenediamine (PPD)
Batch:	/
Purity:	$\geq$ 99%
Dose:	30 mg/kg/d PPD
Route:	Intraperitoneal injection
Exposure:	5 days
GLP:	not in compliance

Five day old male and female NMRI mice (51 males and 55 females) were injected intraperitoneally with 30 mg/kg/d PPD for 5 days. Positive control animals received 300 mg/kg/d urethane and vehicle control animals received 10 mg/kg/d soy bean oil. Total observation time was 130 weeks.

Treatment with PPD did not affect survival or bodyweight. Tumours occurred in 30.1% of the PPD-treated animals as compared to 18.2% in vehicle control animals and 82.1% in urethane-treated animals. The most commonly observed tumours were lymphomas and alveolar adenomas in all groups. The incidence of these tumours (both sexes) is shown in Table 1.

Table 1: Tumour incidence

Tumour type	Vehicle control (10 ml/kg/day)	PPD (30 mg/kg/day)	Urethane (300 mg/kg/day)
Lymphoma	10.4%	18.3%	12.5%
Alveolar adenoma	9.1%	10.8%	76.8%

PPD exposure did not change the frequency of lymphomas ($P>0.10$) or alveolar adenomas ($P>0.37$). A slight, statistically significant increase in overall tumour incidence was calculated for PPD-treated males ($p=0.03$).

Ref.: 51

Comment
Nine studies on PPD alone were identified. The studies involved oral administration, topical administration, as well as injections. Most of the studies were old and the quality varied. One study with intraperitoneal injection of female strain A mice (highest dose, lung tumours) and one study with intraperitoneal injection of neonatal male NMRI mice (overall tumour incidence) were positive. All the other studies, and most important an US NTP study with oral administration of PPD, were negative.

p-Phenylenediamine and hydrogen peroxide

Topical administration, mice

Guideline:	/
Species/strain:	Swiss-Webster mice
Group size:	100 (50 Males and 50 females) per treatment group and vehicle control
Test substance:	p-Phenylenediamine (PPD). Three hair dye formulations (PP-7588, PP-7586, PP-7585) containing 1.5% PPD prior to mixing with equal volume 6% hydrogen peroxide just prior to use.
Batch:	Lot 1143
Purity:	>99%
Dose:	0.05 ml of a solution containing PPD and hydrogen peroxide
Route:	Topical, 1 application weekly and 1 application every second week
Exposure:	18 months
GLP:	not in compliance

Three oxidation hair dye formulations containing 1.5% PPD, mixed with an equal volume of 6% hydrogen peroxide, were tested by topical application in groups of 100 mice weekly or once every two weeks for 18 months. 7,12-dimethylbenz(a)anthracene (DMBA) (0.1%) and were kept as positive controls (0.05 ml containing 50 µg DMBA first 60 months, 10 µg next 4 months and 50 µg last 8 months). The mice were observed daily for signs of toxicity. Each week they were weighed, the skin graded for irritation and papillomas and other gross lesions were noted. Animals that died or that were killed because of general debility were autopsied and examined histopathologically when possible. At termination of the study, all survivors were weighted and killed and a gross autopsy was performed.

There were no overt sign of systemic toxicity in any of the dye-treated groups. The survival varied from 58 to 80% except in the positive controls in which only 21% of the mice were alive after 18 months. Average body weights were comparable in all groups throughout the study.

It is concluded that no evidence of carcinogenic activity was seen.

Ref.: 19

Guideline:	/
Species/strain:	Swiss-Webster mice
Group size:	28 Males and 28 females per treatment group and positive control, 14 males and 14 females in vehicle control group and 76 males and 17 females in untreated control group
Test substance:	p-Phenylenediamine (PPD). Two hair dye formulations containing 1.5% PPD prior to mixing with equal volume 6% hydrogen peroxide
Batch:	/
Purity:	not given
Dose:	0.05 ml of a solution containing PPD and hydrogen peroxide
Route:	Topical, 1 application weekly
Exposure:	2 years
GLP:	not in compliance

Two oxidation hair dye formulations containing 1.5% PPD, mixed with an equal volume of 6% hydrogen peroxide, were tested by topical application in groups of 28 male and 28 female mice weekly for 2 years. 7,12-dimethylbenz(a)anthracene (DMBA) (0.1%) and were kept as positive controls (0.05 ml containing 2.5 and 10 µg DMBA). Each week they were weighed. When signs of marked irritation, ulceration, or tumour formation were evident, the application of the chemical was discontinued until the skin looked "normal". Tissue specimens were taken from all major organ systems and tumours of mice found dead during the study or sacrificed when moribund or at 2 year, the termination of the experiment.

Body weight gains of mice in treated groups were not significantly different from those of mice in the untreated control groups.

It was concluded that the male and female mice in all groups developed both malignant and benign neoplasms. There were no statistical difference between the sexes in the total number of neoplasms or in the incidence of neoplasms of a particular organ and type. The incidence of skin neoplasms did not show statistically significant differences in any of the groups under test except for the positive control groups exposed to DMBA.

Ref.: 42

Guideline:	/
Species/strain:	Swiss-Webster mice
Group size:	50 animals per sex and dose
Test substance:	Four hair dye formulations, 7406 containing 4% p-phenylenediamine (PPD), 7401 containing 3% PPD, 7402 containing 2% PPD, and P-21 containing 1% PPD prior to mixing with equal volume 6% hydrogen peroxide. The mixture was used within 15 minutes after mixing
Batch:	/
Purity:	not stated
Dose:	0.05 ml of a solution containing PPD and hydrogen peroxide
Route:	Topical, 1 application weekly
Exposure:	21 months: 7401, 7402, and 7406 23 months: P-21
GLP:	not in compliance

The experiment involved 12 treatment groups and 3 negative control groups.

Dye applied topically to a 1 cm^2 area on a clipped (24 hours prior to application) site in the interscapular region. Mice received a dose of 0.05 ml topically without occlusion once weekly from 8 – 10 weeks of age for 21 – 23 months. The animals were observed daily for mortality and signs of toxicity, and were weighed monthly. A continuous weekly record was maintained for any skin lesions noted. After 9 months of treatment, 10 males and 10 females per group were necropsied and the study was terminated after 21 – 23 months. Skin and internal organs were evaluated histologically.

4 – 8 males and 10 –13 females survived to 21 months in the groups receiving the oxidative formulation containing PPD. At 21 months, there were 9-12 males and 11-14 females surviving in the control groups. There were no significant differences in absolute or relative liver or kidney weights in groups of 10 male and 10 female mice necropsied after 7 and 9 months. There were

no statistically significant differences in the distribution of tumours among treated and control groups.

Ref.: A and B

Topical administration, rats

Guideline:	/
Species/strain:	Male and female weanling Sprague Dawley rats, 60 per sex per group
Group size:	60 animals per sex and dose
Test substance:	Four hair dye formulations, 7406 containing 4% p-phenylenediamine (PPD), 7401 containing 3% PPD, 7402 containing 2% PPD, and P-22 containing 1.0% PPD prior to mixing with equal volume 6% hydrogen peroxide. The mixture was used within 15 minutes after mixing
Batch:	/
Purity:	not stated
Dose:	0.5 ml of the test substance
Route:	Topical: 1 application twice weekly
Exposure:	114 weeks
GLP:	not in compliance

The experiment involved altogether ten different dye formulations and six control groups.

Groups of 60 male and 60 female were obtained from the first mating (F_{1a}) of a multi-generation reproduction study in rats treated with four different hair dye formulations containing up to 2% PPD. The F_0 parents had received topical application of the hair dye formulation from the time of their weaning to the weaning of their offspring. The dye formulations were administered topically to the shaved (24 hours prior to application) neck and back area twice weekly. An initial dosage level of 0.2 ml/rat was increased incrementally by 0.1 ml per week until 0.5 ml was achieved. There were three independent control groups each containing 60 males and 60 females, which received no treatment.

The rats were observed daily for overt signs of toxicity and for mortality. Detailed observations were recorded weekly. Individual body weights were recorded weekly for the first 14 weeks and monthly thereafter. Group food consumption was recorded weekly. Haematological, biochemical and urinalysis studies were done on 5 males and 5 females per group at 3, 12, 18, and 24 months of study. After 12 months of treatment, 5 males and 5 females from each group were sacrificed and necropsied. Histopathological evaluations were performed on 18 tissues (plus tumour masses) including treated skin.

Survival just prior to terminal sacrifice (at week 114) the survival was 20 – 24 males and 22 – 25 females for the exposed groups. Survival was 17 – 20 males and 22 – 26 females for the control groups. After 114 weeks, group mean body weights in the treated groups were 660 – 678 g in males and 436 – 473 g in females. Control group values ranged from 682 to 759 g in males and 477 to 513 g in females.

There were no significant changes in haematological values in the treated groups at 18 and 24 months. No significant differences considered to be treatment related were observed in the biochemical studies or in the urinalysis. Non-neoplastic lesions were those commonly found in ageing rats and were considered to be spontaneous. The incidence of pituitary adenomas in the

females of group 7406 was significantly higher than in all three control groups, but the high background incidence of this lesion casts doubt on the biological significance of this finding. It was concluded that no increased tumour incidence were found in any of the tissues examined.

Ref.: 26, B, C

Topical administration and subcutaneous injection

Guideline:	/
Species/strain:	Wistar rats
Group size:	10 Males and 10 females
Test substance:	p-Phenylenediamine (PPD)
Batch:	/
Purity:	not stated
Dose:	Topical: Group 1; 0.5 ml of a 1:1 mixture of 5% PPD (in 2% NH_4OH) and 6% H_2O_2 once a week for 18 months Subcutaneous injection: Group 2; 0.1 ml of a 1:1 mixture of 5% PPD (in 2% NH_4OH and 1.8% NaCl) and 6% H_2O_2
Route:	Topical or subcutaneous injection
Exposure:	Topical: 1 weekly application for 18 months Subcutaneous injection: 1 injection every other week for 18 months
GLP:	not in compliance

Wistar rats, a total of 40 males and 40 females were divided into four groups (10 males and 10 females). Group 1 was painted on shaved skin on the back with 0.5 ml of a 1:1 mixture of 5% PPD (in 2% NH_4OH) and 6% H_2O_2 once a week for 18 months. Group 2 was given s.c. injection of 0.1 ml of a 1:1 mixture of 5% PPD (in 2% NH_4OH and 1.8% NaCl) and 6% H_2O_2 at the hips every other week for 18 months. Groups 3 and 4 received topical application and s.c. injection respectively, with corresponding vehicles only and served as controls.

Application to skin

The topical application resulted in a slight decrease in the bodyweights of the males after 30 weeks of exposure. No such effects were found among the females. The total PPD exposure of the rats during the eighteen months of treatment was 975 mg (12.5 mg/treatment). 40% (4/10) of the males developed tumours (1 cholangiocarcinoma and 1 adenoma of the liver, 1 nephroblastoma with lung and pancreas metastasis, 1 cortical adenoma of adrenal gland) and 60% (6/10) females developed tumours (1 fibromatosis and 5 mammary gland tumours which include fibrosarcoma, fibroadenoma and adenoma). The first mammary tumours in the female rats were observed after 47 weeks. The others were observed after 49, 60, 72 and 85 weeks. No tumour was found in the 10 male control rats, while 1 tumour (stromal cell sarcoma of the uterus) was found in the female control rats.

Subcutaneous injection

The total PPD dose during the eighteen months was 97.5 mg in the s.c. study. One male rat (14.3%; 7 effective animals) developed both a follicular carcinoma of thyroid and undifferentiated carcinoma of the lung. Among the 7 female rats, 6 (85.7% developed tumours, 4 soft tissue tumours (includes unclassified sarcoma and lipoma) and 4 mammary gland tumours (ductectasia or adenosis), and 3 uterus tumours (includes adenocarcinoma, endometrial polyp and glandular cystic hyperplasia). No tumours were found among the control animals.

Table 2: The incidence of tumours

Group	Treatment	Number of rats	Percent tumour bearing	Number of rats bearing tumours in tissue				
				Soft tissue	Mammary gland	Uterus	Liver	'other'
Males								
1	PPD/HP dermal	10	40.0	0	0	-	2a	2b
2	PPD/HP SC	7	14.3	0	0	-	0	1d
3	control dermal	10	0.0	0	0	-	0	0
4	control SC	9	0.0	0	0	-	0	0
Females								
1	PPD/HP dermal	10	60.0	1c	5*	0	0	0
2	PPD/HP SC	7	85.7	4e*	4*	3f*	0	0
3	control dermal	9	11.1	0	0	1g	0	0
4	control SC	10	0.0	0	0	0	0	0

HP = Hydrogen peroxide SC = Subcutaneous * p= < 0.05 vs. controls. a – Cholangiocarcinoma and a hepatic adenoma. b – Nephroblastoma with lung/pancreatic metastases, transitional cell papilloma of bladder, with cortical adenoma of adrenal gland. c – Stromal cell sarcoma. d – Thyroid follicular carcinoma and lung carcinoma (undifferentiated). e – includes 'unclassified sarcoma and lipoma'. f – Includes adenocarcinoma, endometrial polyp and glandular cystic hyperplasia. g - Stromal cell sarcoma

The authors pointed out that it is of particular interest to note that the mammary gland of female rats was the primary target organ of the oxidation product of PPD. This organ has also been shown to be highly susceptible to tumour induction by 2,4-diaminotoluene and a wide range of N-substituted aromatic amines. It is concluded that oxidized PPD induced a statistically significant incidence of mammary gland tumours both after topical application and after subcutaneous injection in female rats.

Ref.: 97

Guideline: /
Species/strain: Wistar rats
Group size: 10 Males and 10 females
Test substance: p-Phenylenediamine (PPD)
Batch: /
Purity: not stated
Dose: See Table 3
Route: topical or subcutaneous injection
Exposure: Topical: 1 weekly application for 18 months
Subcutaneous injection: 1 injection every other week for 18 months
GLP: not in compliance

The study was conducted in Wistar rats (n = 10/sex/group). Three PPD-containing samples were tested by the topical route, two of which contained resorcinol at levels of 3.25 or 2.28 %, and a third sample contained PPD only at 5.93 % in water. All samples were mixed with hydrogen peroxide prior to dermal application, once weekly for 18 months. The study also included groups dosed by the subcutaneous route. The same formulations as used for topical application were injected every 2 weeks for 18 months. In addition, a sample comprising simply PPD powder mixed with saline (125 mg PPD/ml) was also included in the s.c. injection experiment of this

study. Control animals were dosed with the appropriate vehicle (3% hydrogen peroxide, 0.9 % NaCl and 2% NH_4OH). All groups were terminated after 24 months.

Type and composition of the formulations are given in Table 3.

Table 3: Types and chemical composition of hair dyes

Group	Type	Chemical composition	Content (%)
1	Water	Control	
2	Water	PPD Resorcinol	4.65 3.25
3	Water	PPD Resorcinol	4.32 2.28
4	Water	PPD	5.93
5	Powder	PPD (only s.c. injection)	125 mg/ml

For application to skin, the hair dyes were oxidised with 6% hydrogen peroxide at the ratio 1:1 by volume or weight 20-30 min before test. For subcutaneous injection, hair dyes were oxidised with 6% hydrogen peroxide at the ratio 1:1 by volume and NaCl added to a concentration of 0.9% in the final oxidation mixture. Sample 5 was mixed with water to a final concentration of 125 mg/ml and NaCl added. Control group comprised 3% hydrogen peroxide, 0.9% NaCl and 2% NH_4OH.

Application to skin
Groups of 10 males and 10 females Wistar rats were painted once a week for 18 months. The experiment was terminated after 24 months. The treatment did not influence the weight of the animals. The tumours induced are shown in Table 4.

Table 4: Incidence of tumours in rats treated with hair dyes by skin painting

Group	Treatment	Number of rats	Percent tumour bearing	Number of rats bearing tumours in tissue				
				Soft tissue	Mammary gland	Uterus	Liver	'other'
Males								
1	Control	10	0.0	0	0	-	0	0
2	PPD/Res 3.25%	10	20.0	1	0	-	0	1a
3	PPD/Res 2.28%	9	22.0	0	0	-	0	2b
4	PPD alone	10	20.0	1	1	-	0	0
Females								
1	Control	9	11.0	0	0	1	0	0
2	PPD/Res 3.25%	9	89.0	3	6*	1	0	1a
3	PPD/Res 2.28%	10	60.0	0	4	2	0	0
4	PPD alone	10	60.0	0	5*	1	0	1a

* p= < 0.05 vs. controls. a –Thyroid tumour. b – Kidney transitional cell carcinoma and bronchial adenoma. Res-Resorcinol

Subcutaneous injection

Groups of 10 males and 10 females Wistar rats received subcutaneous injection in the back hip every 2 week for 18 months. The experiment was terminated after 24 months. The treatment did not influence the weight of the animals. The tumours induced are shown in Table 5.

Table 5: Incidence of tumours in rats treated with hair dyes by subcutaneous injections.

Group	Treatment	Number of rats	Percent tumour bearing	Number of rats bearing tumours in tissue				
				Soft tissue	Mammary gland	Uterus	Liver	'other'
Males								
1	Control	9	0.0	0	0	-	0	0
2	PPD/Res 3.25%	9	67.0	5*	1	-	0	0
3	PPD/Res 2.28%	8	50.0	4*	0	-	0	0
4	PPD alone	9	22.0	0	0	-	1	1a
5	PPD powder	10	20.0	1	1	-	0	0
Females								
1	Control	10	0.0	0	0	0	0	0
2	PPD/Res 3.25%	9	78.0	4	2	0	0	1a
3	PPD/Res 2.28%	10	60.0	3	5*	0	1	1b
4	PPD alone	9	100.0	4	8*	0	0	3c
5	PPD powder	9	56.0	2	3	0	0	1b

* p= < 0.05 vs. controls. a- Salivary gland tumour. b- Thyroid tumour. c- Thyroid, glandular cystic hyperplasia of endometrium and hypertrophy. Res- Resorcinol

The authors conclude that all 5 samples of hair dyes caused benign and malignant tumours in various organs of exposed rats in both sexes. Particularly, soft tissue tumours were increased in both sexes and mammary gland tumours were increased in female rats. The mammary gland tumours found in some groups of rats were malignant type.

Ref.: 98

Comment

The sensitivity of animal studies to detect carcinogenic substances is rather low. Thus, in experiments with groups containing 50 animals, the tumour incidence has in most cases to increase by more than 5% in order to be acknowledged. As a consequence, the animals are generally exposed to amounts of chemicals that are much higher than that humans normally are exposed to. In the studies of PPD together with hydrogen peroxide, the exposure has been less or comparable to the exposure of humans using hair dyes.

Six studies on PPD together with hydrogen peroxide were identified. Three of the studies involved topical application of Swiss-Webster mice. All 3 studies tested hair dye formulations that also contained 2,4-toluenediamine and/or 2,4-diaminoansole. It should be noted that these dyes are classified as a carcinogen category 2 in EU. The use of 2,4-toluenediamine was banned in hair dyes in EU in 1980 and 2,4-diaminoanisol was banned in 1986. In spite of the fact that in all three studies involved known carcinogenic substances, no tumours related to the treatment was detected.

Three of the studies on PPD together with hydrogen peroxide involved topical application of rats. One study from USA used Sprague Dawley rats that were painted with hair dye formulations containing up to 2% PPD. In the same study, ten different hair dye formulations

were tested. Although some of the formulations contained 2,4-diaminoanisol, none of the formulations induced tumours in the rats.

A group at the National Cancer Institute in Thailand published in 1986 two of the studies. In both studies, there was a statistically significant increase in mammary gland tumours in female rats. The studies did also involve subcutaneous injection of the same mixtures. Also in the s.c. injection studies a statistically significant increase in mammary gland tumours in female rats was found. In addition, local soft tissue tumours were induced. The latter tumours are, however, not considered relevant for humans. The studies have been criticized. Only one tumour was reported in the control groups. It looks like the control groups may have been the same in the two studies. The group size was rather small, only 10 animals. Possible explanations for the discrepancy between the two studies from Thailand and the study from USA may be different sensitivity of Sprague Dawley rats and Wistar rats. Moreover, hair dye formulations were used in the study from USA while the studies from Thailand involved mixtures of PPD with hydrogen peroxide or PPD + resorcinol + hydrogen peroxide. Furthermore, the concentrations of PPD seem to have been a little higher in the studies from Thailand than in the study from USA.

IARC has classified PPD as a category 3 carcinogen based on no data in human studies and inadequate data in animal studies. This classification was carried out in 1978. PPD is classified by Germany (MAK) as a category 3 B carcinogen.

Conclusion
p-Phenylenediamine alone has not been demonstrated to be carcinogenic in experimental studies with rats or mice.

Hair dye formulations of p-phenylenediamine together with hydrogen peroxide have not been demonstrated to be carcinogenic in experimental studies after topical application to mice. The sensitivity of these studies may have been low as they did not respond to hair dye formulations containing known carcinogens. Thus, no conclusions with regard to carcinogenicity can be drawn from the studies.

Hair dye formulations of p-phenylenediamine together with hydrogen peroxide have been tested in three experimental studies after topical application to rats. The sensitivity of one of these studies may have been low as no response to hair dye formulations containing known carcinogens was observed. Thus, no conclusions with regard to carcinogenicity can be drawn from this study.

In two studies, mixtures of p-phenylenediamine and hydrogen peroxide or p-phenylenediamine and resorcinol and hydrogen peroxide were tested by topical application as well as by subcutaneous injection of rats. In both studies a statistically significant increase mammary gland tumours were found both after topical application as well as after subcutaneous injection. The studies have been criticized. However, it is not possible to disqualify the studies completely.

p-Phenylenediamine together with hydrogen peroxide may be carcinogenic in experimental studies with rats.

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

Taken from SCCNFP/0129/99, adopted in 2002

A two-generation reproduction toxicity study has been performed in the male Sprague Dawley Rat (40 males and 40 females per group, 3 control groups). Hair dye formulations containing 2%, 3% or 4% PPD were mixed with an equal volume of hydrogen peroxide and applied topically to the backs and necks of the animals twice a week (generation F_0).

Treatment was continuous through growth, mating, gestation and lactation to the weaning of the F_{1A} and F_{2B} litters. No compound-related effects on survival, general appearance, food consumption, body weight gain, fertility of males or females or on gestation, lactation or weaning indices were observed.

Ref.: 26

3.3.8.2. Teratogenicity

Taken from SCCNFP/0129/99, adopted in 2002

PPD had been tested in one group of 25 Mice receiving a subcutaneous dose of 28 mg/kg in aqueous solution on days 5 to 7, 8 to 10 or 11 to 14 of gestation. No teratogenic effect was observed.

Ref.: 96

Four hair dyes formulations containing 1, 2, 3 or 4% PPD were rinsed with hydrogen peroxide before topical application at 2 ml/kg (corresponding to 20, 40, 60 and 80 mg/kg) to groups of 20 mated female rats on days 1, 4, 7, 10, 13, 16 and 19 of gestation.
No significant differences were found between control and treated group. No teratogenic activity was observed.

Ref.: 20

Hair dyes formulations containing 3% PPD mixed with an equivalent solution of hydrogen peroxide were applied topically twice a week to female rats, 4 weeks prior mating and throughout mating and gestation.
No evidence of maternal toxicity or teratogenic effect was observed.

Ref.: 13

PPD was administered by gavage to pregnant female Sprague Dawley rats on day 6 through 15 of gestation at the dose levels of 5, 10, 15, 20 or 30 mg/kg/day. Pregnant animals were killed on day 20 of gestation; visceral and skeletal malformations were recorded on the foetuses.
Significant maternal toxicity was observed at 20 and 30 mg/kg/day (reduced body weight gain and decreased food consumption). No biologically or statistically significant increase in malformations or developmental variations was observed at any dose level.
Under the experimental conditions adopted, PPD revealed no teratogenic or embryo-toxic effects.

Ref.: 93

A one-generation reproduction toxicity study has been performed in the male Sprague Dawley Rat. 0.5 ml of hair dyes, one corresponding to a semi-permanent and the other to an oxidative dye, both containing 2.2 % PPD (approximately 11 mg/kg/day) was applied topically to the

backs of 25 rats twice a week for 10 weeks. After the treatment period, each male (P_0) was mated to 1 female each week for 3 weeks. One hundred F1 male offspring from these matings were mated to 1 female per week for 3 weeks. Female rats were killed between day 14 and 16 of gestation. There were no compound-related effects observed on P_0 male body weight gains, percent fertility or total and average live pups per F1 litter. No evidence of reduced fertility was recorded in the F1 males. No compound-related changes were noted in the number of implantations, dead foetuses and resorptions.

Ref.: 24

Prenatal development toxicity study

Guideline:	OECD 414 (2001)
Species/strain:	Sprague-Dawley rats, strain Crl: OFA (SD)
Group size:	25 pregnant females per dose group
Test substance:	p-phenylenediamine
Batch:	1365
Purity:	99.8%
Dose levels:	0, 5, 10, or 20 mg/kg bw/day in water
Treatment:	once daily at days 6 - 19 of gestation by gavage
GLP statement:	In compliance

The test item was daily freshly prepared as solution in deionised water which was degassed before by sonication and saturated with nitrogen gas and kept under nitrogen atmosphere for approximately 15 min. Stability and homogeneity was checked by HPLC.
Groups of 25 pregnant rats received the test substance by gavage at doses of 0, 5, 10 or 20 mg/kg bw/day from day 6 through day 19 of gestation, the control group received the vehicle only (water). The day of positive proof for sperm in the vaginal smear was designated as day 0 of pregnancy.

Animals were checked daily for clinical signs. Food consumption and body weight were recorded at designated intervals during pregnancy (days 0-6, 6-9, 9-12, 12-15, 15-18, and 18-20). On day 20 of pregnancy, the animals were killed and examined macroscopically. Foetuses were removed by Caesarean section. The following litter parameters were recorded: number of corpora lutea and implantation sites, number and distribution of early and late resorptions, and number and distribution of dead and live foetuses, uterus weight and sex. Foetuses were weighed and submitted to external, soft tissue (one half) and skeletal examinations.

Results
No treatment-related effects in dams were noted with regard to clinical observations and post-mortem findings. Body weight gain was transiently lower during the first 3 days of treatment at 10 and 20 mg/kg bw, food consumption was not affected. At 10 and 20 mg/kg the mean net body weight changes were decreased.

Foetal body weight was lower at 20 mg/kg bw, but the difference was statistically not significant. At this dose also retardation in ossification was seen (supraoccipital, sternebrae, thoracic vertebrae, metacarpals).

Conclusion

The NOAEL of maternal toxicity is 5 mg/kg bw whereas the NOAEL of developmental toxicity is 10 mg/kg bw.

Ref.: 8, subm. 12/2005

3.3.9. Toxicokinetics

Human hepatic metabolism in vitro

Guideline:	/
Cells:	human hepatocytes, 4 male donors (cryo-preserved)
Test substance:	p-phenylenediamine dihydrochloride Sigma-Aldrich P6001
Radiolabeled substance:	[^{14}C(u)]-1,4-phenylenediamine dihydrochloride (^{14}C PPD)
Specific activity:	33.2 mCi/mmol
Batch:	Lot No. 10194-57-A
Purity:	> 98%
Test system (concentrations):	**A** hepatocytes (10 µM, 50 µM and 100 µM PPD) **B** microsomal preparation (10 µM and 100 µM PPD) **C** recombinant human CYPs (10 µM and 100 µM PPD)
GLP:	/

A The hepatocytes were thawed and pooled and then incubated for 1, 2 or 4 h with 10 µM, 50 µM and 100 µM ^{14}C-labelled p-phenylenediamine (PPD). The reaction was stopped by acetonitrile and analysed by LC/MS/MS.

B-1 10 µM and 100 µM ^{14}C-labelled p-phenylenediamine (PPD) and, in addition, its acetylated metabolites were incubated for 1 h with pooled human liver microsomes in the presence and absence of an NADPH regenerating system and analysed for the formation of mono-oxygenated derivatives. As a positive control 2-aminofluorene (100 µM) was used.

B-2 Human liver microsomes were incubated for 1 h with 40 µM and 130 µM ^{14}C-labelled p-phenylenediamine (PPD) in the presence and absence of an NADPH regenerating system. Radioactivity in the pellets of a 2000 rpm centrifugation was determined by liquid scintillation counting.

C 10 µM and 100 µM ^{14}C-labelled p-phenylenediamine (PPD) were incubated for 1 h with recombinant human CYPs derived from a bacterial expression system (CYP1A1, CYP1A2, CYP1B1, CYP2C9, CYP2C19, CYP2D6 and CYP 3A4).

Results

A After incubation of PPD with human hepatocytes the two acetylated metabolites N-acetyl-p-phenylenediamine and N,N´-diacetyl-p-phenylenediamine were identified.

B-1 After incubation of PPD and its acetylated metabolites for 1 h with pooled human liver microsomes in the presence and absence of an NADPH regenerating system no mono-oxygenated products were found. The activity of the system was proven by the detection of hydroxylated metabolites of 2-aminofluorene.

B-2 No increase in the covalent protein binding of radioactivity in the microsomal fraction was found in the presence of a NADPH regenerating system.

C After incubation with the different CYPs no metabolites were detected. In contrast, 4 hydroxylated metabolites of the positive control 2-aminofluorene were quantified.

Conclusion
The data of the study show that PPD is metabolized by human hepatocytes and microsomes to N-acetylated derivatives but there were no evidence for the formation of mono-oxygenated metabolites by bacterially expressed human CYPs.

Ref.: 2, 3 (subm. 7/05)

Urinary excretion of [^{14}C]-p-phenylenediamine derived metabolites in humans exposed to an oxidative hair dye formulation

Guideline:	/
Test system:	8 male humans
Specific radioactivity:	57 mCi/mmol
Radiochemical purity:	98.2%
Test substance:	2% [^{14}C-PPD], in a hair dye formulation with m-aminophenol after mixing with H_2O_2
GLP:	in compliance

The absorption of a commercial [^{14}C]-PPD-containing oxidative dark-shade hair dye (80 ml, containing 2.0% [^{14}C]-PPD) was investigated in human volunteers. The hair of 8 male volunteers was cut to a standard length, dyed, washed, dried, clipped and collected. Hair, washing water, materials used in the study and a 24-hour scalp wash were collected for determination of radioactivity. Blood, urine and faeces were analysed up to 120 hours after hair dyeing.

Results
The recovery rate was 95.7 ± 1.5% of the applied radioactivity. Washing water, cut hair, gloves, paper towels, caps or scalp wash contained a total of 95.16 ± 1.46% of the applied [^{14}C]. Absorbed radioactivity amounted to 0.50 ± 0.24% in the urine and 0.04 ± 0.04% in the faeces. Within 24 hours after application, most of the radioactivity was eliminated. The C_{max} of [^{14}C]-PPD equivalents in the plasma was 0.087 μg_{eq} / ml, the T_{max} was approximately 2 hours, and the calculated mean $AUC_{0\text{-}24h}$ was 0.98 μg_{eq} / ml * hr.

Conclusion
Derived from the radioactivity found in urine and faeces 0.54 % of radiolabelled PPD is dermally absorbed and excreted within 24 h following application of a 2 % oxidative hair dye formulation on the scalp of humans.

Ref.: 4, 5 (subm. 7/05)

Comment
Binding to macromolecules in the scalp was not investigated.

Human urinary metabolites and human NAT2 genotyping of males exposed to an oxidative hair dye formulation

Guideline: /

Test system:	8 male humans
Specific radioactivity:	57 mCi/mmol
Radiochemical purity:	98.2%
Test substance:	2% [^{14}C-PPD], in a hair dye formulation with m-aminophenol after mixing with H_2O_2
GLP:	in compliance (genotyping excluded)

This study is part of the previous study (Ref. 4 and 5). Urine samples of individuals were collected for 24 h, pooled, concentrated using solid phase columns and analysed by HPLC equipped with a flow scintillation analyzer. NAT2 genotypes were determined from subject DNA gained from 10 ml blood samples using single nucleotid polymorphism (SNP)-specific PCR primers and fluorogenic probes.

Results

Related to the applied radioactivity the mean urinary excretion was 0.43 ± 0.24 %. Related to total urinary radioactivity 44.6 ± 8.9% and 48.9 ± 9.6% were found in 2 peaks which appears to correspond to N-acetyl-p-phenylenediamine and N,N´-diacetyl-p-phenylenediamine.

With regard to the NAT2 genotype 3 of the subjects were classified as slow acetylators, 5 as intermediate acetylators. The comparison of the metabolic profile of the 2 subgroups as given by the rate of acetylated derivatives exhibited no difference.

Ref.: 6, 7 (subm. 7/05)

Biotransformation of p-phenylenediamine in reconstructed human epidermis and human hepatocytes

Guideline:	/
Test system:	**A** reconstructed human epidermis (Episkin®) **B** primary human hepatocytes
Specific radioactivity:	2.2 GBq (59.4 mCi) / mmol
Radiochemical purity:	97.8%
Test substance:	0.5 µCi/ml [14C-PPD], 20 - 1000 µM
GLP:	/

Epidermis or hepatocytes were incubated at 37 °C for 24 h with the test substance. Samples were further processed and analysed by HPLC equipped with a diode array detector and an on-line radioactivity detector. The metabolites were identified by ESI mass spectrometry.

Results

A Reconstructed human epidermis

In reconstructed human epidermis covalent protein binding ranged from 2.4 (20 µM PPD) to 0.5% (1000 µM PPD) of the applied radioactivity per mg protein. At 20 µM PPD 10.5% and 50.3% of the applied were related to N-acetyl-p-phenylenediamine and N,N´-diacetyl-p-phenylenediamine, whereas 20.4% were due to unchanged PPD. The rest of the radioactivity was due to covalent binding (2.4%), protein adsorption (11.5 %) and HPLC noise (7.3%).

B Primary human hepatocytes

In human hepotocytes at 20 µM PPD 3.5% and 82.1% of the applied were related to N-acetyl-p-phenylenediamine and N,N´-diacetyl-p-phenylenediamine, whereas 1.3 % were due to unchanged PPD. The rest of the radioactivity was due to covalent protein binding (0.25%), protein adsorption (1.48%) and HPLC noise 7.5%).

Ref.: 8

Comment

Covalent protein binding in the skin is occurred, DNA binding was not investigated.

Pharmacokinetics of [^{14}C]-p-phenylenediamine in rats following oral administration and plasma metabolites after dermal administration

Guideline:	/
Test system:	Sprague-Dawley rats
Treatment groups:	**A kinetics:** 6 males and 6 females 6.45 mg/kg bw, once by gavage **B excretion:** 3 males and 3 females 6.45 mg/kg bw, once by gavage **C metabolism:** 3 males and 3 females 49.9 mg/kg bw, dermal application (solvent 40% ethanol, occlusive, 4 h)
Test substance:	p-phenylenediamine dihydrochloride in water (exception dermal application 40% ethanol)
Batch:	102K1201
Radio-labelled substance:	p-[U-^{14}C]phenylenediamine dihydrochloride (^{14}C-PPD)
Specific radioactivity:	2.2 GBq (59.4 mCi) / mmol
Batch:	CFQ13408

Purity: 97.8%
GLP: in compliance

The test substance was given by gavage (groups **A** and **B**) or dermally (group **C**) on clipped skin of the interscapular region resembling approximately 20% of the body surface. The test item was left on the skin for 4 h. Blood samples were taken 0.5, 1, 2, 4, 8, 12, and 24 h post-gavage (**A** and **B**) or at 4 h (end of exposure, **C**), respectively. Blood plasma and excreta were analysed for radioactivity by liquid scintillation counting. In group **C** the plasma samples were analyzed for metabolic pattern by radio-HPLC.

Results
A Plasma pharmacokinetics: following oral gavage at 6.45 mg/kg the mean plasma radioactivity levels increased to a C_{max} (7.12 ± 0.18 and 6.88 ± 0.83 µg-eq/ml, in males and females, respectively) at 0.5 hours post-gavage, followed by a regular decrease to low levels at the 24-hour sampling point (0.04 or 0.07 µg-eq/ml for males and females, respectively). The respective plasma AUC_{0-t} values were 24.85 and 27.30 µg-eq * hr / ml.
B Excretion balance: the mean recovery of the administered dose over 24 hours is shown in the following table:

Sex	Urine (%)	Faeces (%)	Cage Wash (%)	Carcass (%)	Total recovery (%)
Male	74.4 ± 5.9	19.3 ± 2.6	3.1 ± 0.7	7.0 ± 0.3	103.8 ± 6.0
Female	81.0 ± 4.3	13.8 ± 1.4	4.8 ± 1.9	4.7 ± 1.0	104.4 ± 1.8

C Metabolism: The radioactivity levels in the plasma collected were 1412 and 7401 ng-eq/g for males and females, respectively which may suggest gender differences in percutaneous absorption. During HPLC separation radioactivity was only found at the retention time of N,N´-diacetyl-p-phenylenediamine.

Ref.: 9

Pharmacokinetics and mass balance of radioactivity in rats following single oral administration of [^{14}C]-p-phenylenediamine

Guideline: /
Test system: Sprague-Dawley rats
Test substance: p-phenylenediamine dihydrochloride in water
Batch: 102K1201
Radiolabeled substance: p-[U-^{14}C]phenylenediamine dihydrochloride (^{14}C-PPD)
Specific radioactivity: 2.2 GBq (59.4 mCi) / mmol
Batch: CFQ13408
Purity: 97.8%
GLP: in compliance

The test substance (4 mg/kg) was applied once to 6 males and 6 females (plasma pharmacokinetics) and 3 males and females (excretion balance) by gavage. Blood samples were taken at 0.5, 1, 2, 4, 8, 12, and 24 h post-gavage (kinetics), urine and faeces were collected during 24 h (excretion). Blood plasma and excreta were analysed for radioactivity by liquid scintillation counting.

Results
Plasma pharmacokinetics: following oral administration, mean plasma radioactivity levels increased rapidly to Cmax values (4.10 ± 0.04 or 3.73 ± 0.23 µg/ml for males and females, respectively) at 0.5 hours post-gavage, followed by a regular decrease to the last sampling point at 24 hours, i.e. 0.015 and 0.022 µg/ml, respectively. The respective plasma AUC_{0-t} values were 10.84 or 10.80 µg * hr / ml.

Excretion balance: the mean recovery of radioactivity over a 24-hour period is shown in the following table:

Sex	Urine (%)	Faeces (%)	Cage Wash (%)	Carcass (%)	Total recovery (%)
Male	57.0 ± 3.1	23.7 ± 2.1	7.3 ± 0.9	3.7 ± 0.3	91.8 ± 2.9
Female	60.1 ± 3.4	19.3 ± 1.5	8.3 ± 1.0	4.2 ± 0.6	92.0 ± 1.1

Conclusion
Following oral administration, the plasma pharmacokinetics show a rapid absorption phase (t_{max} = 0.5 hours) followed by excretion which was nearly complete at 24 hours. The C_{max} and plasma AUC values suggest good bioavailability and minimal inter-animal or gender-related variability
Ref.: 10

Summary of studies on toxicokinetics

Two rat studies *in vivo* show that PPD after oral administration is quickly absorbed and excreted to a large extent within 24 h. After dermal application only the diacetylated metabolite was found in blood plasma. Following oral administration of 4 mg/kg, mean plasma radioactivity levels increased rapidly to C_{max} values (4.10 ± 0.04 or 3.73 ± 0.23 µg/ml for males and females, respectively) at 0.5 hours post-gavage, followed by a regular decrease to the last sampling point at 24 hours, i.e. 0.015 and 0.022 µg/ml, respectively. The respective plasma AUC_{0-t} values were 10.84 or 10.80 µg * hr / ml.
Following application of an oxidative hair dye formulation containing radiolabelled PPD to humans in 2 *in vivo* studies ca. 0.4 and 0.5% of the applied radioactivity was found in the urine, and most of the radioactivity was eliminated within 24 h. The C_{max} of [^{14}C]-PPD equivalents in the plasma was 0.087 μg_{eq} / ml, the T_{max} was approximately 2 hours, and the calculated mean AUC_{0-24h} was 0.98 μg_{eq} / ml * hr. More than 90% of the total urinary radioactivity was related to the mono- and diacetylated metabolite of PPD. No difference of the metabolic profile was found in the NAT2 genotype subgroups (low and intermediate acetylators).

Human hepatocytes and microsomes *in vitro* were found to form the 2 acetylated PPD derivatives. In microsomes, no experimental clues were seen with regard to the formation of monoxygenated products of PPD and its acetylated derivatives. Following incubation of PPD with recombinant human CYPs derived from a bacterial expression system no evidence for the formation of mono-oxygenated metabolites was found. In microsomes no increase in covalent protein binding was found.

In a reconstructed human epidermis after 24 h incubation with [^{14}C] PPD 60.8% of the radioactivity was related to the 2 acetylated derivatives and 20.4% to unchanged PPD. whereas 2.4 % was covalently bound to protein.

Generally, the formation rate of the mono- and diacetylated derivatives and the ratio between those vary depending on the test system and the concentration investigated.

3.3.10. Photo-induced toxicity

3.3.10.1. Phototoxicity / photoirritation and photosensitisation

/

3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity

/

3.3.11. Human data

/

3.3.12. Special investigations

/

3.3.13. Safety evaluation (including calculation of the MoS)

Taken from SCCNFP/0129/99, adopted in 2002

CALCULATION OF THE MARGIN OF SAFETY
(p-PHENYLENEDIAMINE)
(Oxidative)

The maximum concentration of 4.0% of p-Phenylenediamine is mixed before use with H_2O_2. Thus the usage volume of 100 ml contains at maximum 2.0%

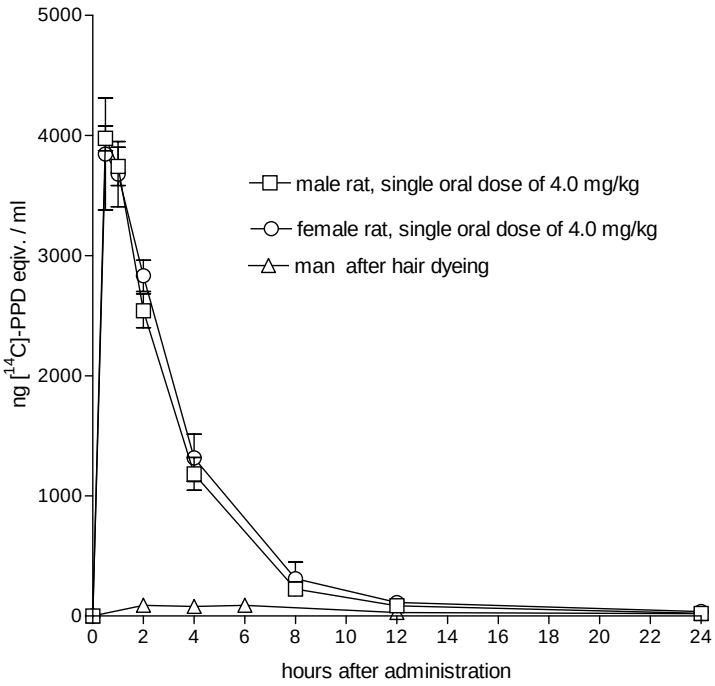
Highest penetration	**PA (μg/cm^2)**	**=**	**4.47 μg/cm^2**
Typical body weight of human		**=**	**60 kg**
Exposed area (scalp)		**=**	**700 cm^2**
Systemic exposure	**PA x 700 cm^2**	**=**	**3.129 mg**
Systemic exposure dose (SED)	**PA x 700/ 60 x 1000**	**=**	**0.052 mg/kg bw**
No observed adverse effect level (mg/kg) (rat, oral, subchronic toxicity)	**NOAEL**	**=**	**4 mg/kg bw**
Margin of Safety	**NOAEL / SED**	**=**	**77**

Safety evaluation based on toxicokinetics (taken from dossier)

A single oral administration of [^{14}C]-PPD to male and female rats of a dose corresponding to the NOAEL of the 90-day oral toxicity study (4.0 mg/kg) produced a measured systemic exposure characterised by plasma C_{max} values for both sexes of approximately 3.9 µg-eq/ml (4.10 ± 0.04 and 3.73 ± 0.23 µg/ml in males and females, respectively) and a mean plasma $AUC_{0\text{-}24h}$ of approximately 10.8 µg-eq * hr / ml.

In human volunteers (N=8), hair dyeing with a dark-shade, 2.0% [^{14}C]-PPD-containing hair dye produced measurable plasma values with a C_{max} of 0.087 µg-eq/ml and a mean $AUC_{0\text{-}24h}$ of 0.66 μg_{eq} * hr / ml (mean value of individual AUCs). Comparison of the human plasma AUC with that of rats at the NOAEL of a subchronic oral toxicity study (4.0 mg/kg/day) yields a toxicokinetic-based safety margin of **16.3-fold.**

Figure 1: Comparison of plasma concentrations ([^{14}C]-PPD µg-eq / ml) in rats after a single dose of 4.0 mg/kg [^{14}C]-PPD with those in human subjects after hair dyeing with a dark shade, 2.0% [^{14}C]-PPD-containing hair dye.



Today there is a general agreement that an AUC-based margin of 4.3 covers the 95% percentile of inter-species differences for toxicokinetics and toxicodynamics (Reference 14). Therefore, the AUC-based margin of safety for PPD is sufficiently high to conclude that the human systemic exposure from hair dyeing with a dark-shade, PPD-containing oxidative hair dye poses no risk to human health. This view is further supported by the margin of safety on the basis of human and rat C_{max} values (45-fold), which gives additional assurance that human systemic exposure from PPD-containing hair dyes is far lower than that in rats at the NOAEL.
Taking into account that the human study was performed under maximal exposure conditions, such as maximal PPD concentration (2.0%), short hair length (2 cm) of the treated subjects and

the actual human exposure to hair dyes is intermittent (4- to 6-week intervals), whereas the subchronic animal study used daily oral administration by bolus gavage, these considerations provide additional assurance and suggest that the human health risk is likely to be negligible.

Comment of the SCCP

According to WHO the 100-fold uncertainty factor can be subdivided in interspecies differences (10-fold) in toxicodynamics (2.5) and toxicokinetics (4) and inter-individual differences (10-fold) in toxicodynamics (3.2) and toxicokinetics (3.2). Given the AUC figures obtained from rats and humans the 4-fold factor for interspecies differences in toxicokinetics can be set to 1 which results in a remaining safety factor of 25 which was not achieved (a factor of 16.3 was calculated).

Ref.: D, E

3.3.14. Discussion

Physico-chemical specifications
No data on stability in test solutions and in marketed products was submitted. Calculated values of log P_{ow} cannot be accepted as estimates of the true physical constants without justification, indicating that the reported values are realistic. Solubility in water is not properly characterised.

General toxicity
In an acute oral toxicity study in rats the animal treated with 100 mg/kg bw died 90 min after dosing. 1 of 2 animals treated with 75 mg/kg died within 3 h. The animal treated with 50 mg/kg bw showed clinical signs (lachrimation, swelling of conjunctivae, gait, tremor, subdued behaviour and/or pilorection). At 25 mg/kg bw only orange traces in the bedding were seen probably due to coloured urine.

Several studies on systemic toxicity have shown that the most sensitive target organ is skeletal muscle, with a rhabdomyolysis being experimentally observed following oral application in the rat at levels down to 10 mg/kg bw. From a 90 day study, a NOAEL of 4 mg/kg bw was obtained and is used as the basis for the safety evaluation.

In a gavage developmental toxicity study in rats the NOAEL of maternal toxicity is 5 mg/kg bw whereas the NOAEL of developmental toxicity is 10 mg/kg bw.

Irritation, sensitisation
PPD was not irritant or corrosive for the skin and the eye when applied in a 2.5% aqueous solution. PPD is an extremely potent contact allergen, both experimentally and in clinical experience.

Dermal absorption
Several relevant studies have been performed on percutaneous absorption of PPD. The highest cumulative penetration obtained was 4.47 $\mu g/cm^2$.

Toxicokinetics
Two rat studies *in vivo* show that PPD after oral administration is quickly absorbed and excreted to a large extent within 24 h. After dermal application only the diacetylated metabolite was

found in blood plasma. Following oral administration of 4 mg/kg, mean plasma radioactivity levels increased rapidly to C_{max} values (4.10 ± 0.04 or 3.73 ± 0.23 µg/ml for males and females, respectively) at 0.5 hours post-gavage, followed by a regular decrease to the last sampling point at 24 hours, i.e. 0.015 and 0.022 µg/ml, respectively. The respective plasma AUC_{0-t} values were 10.84 or 10.80 µg * hr / ml.

Following application of an oxidative hair dye formulation containing radio-labelled PPD to humans *in vivo* ca. 0.4 and 0.5% of the applied radioactivity was found in the urine, respectively, and most of the radioactivity was eliminated within 24 h. The C_{max} of [^{14}C]-PPD equivalents in the plasma was 0.087 μg_{eq} / ml, the T_{max} was approximately 2 hours, and the calculated mean AUC_{0-24h} was 0.98 μg_{eq} / ml * hr. More than 90% of the total urinary radioactivity was related to the mono- and diacetylated metabolite of PPD. No difference of the metabolic profile was found in the NAT2 genotype subgroups (low and intermediate acetylators).

Human hepatocytes and microsomes *in vitro* were found to form the 2 acetylated PPD derivatives. No experimental clues were seen with regard to the formation of monoxygenated products in microsomes as well as in recombinant human CYPs from bacteria. In microsomes no increase in covalent protein binding was found.
In a reconstructed human epidermis after 24 h incubation with [^{14}C] PPD 60.8% of the radioactivity was related to the 2 acetylated derivatives and 20.4% to unchanged PPD. whereas 2.4% was covalently bound to protein.

The results of metabolism studies in human skin and plasma analysis after topical administration to rats suggest that topically applied PPD is converted in human and animal skin to N-mono- or N,N'-diacetylated metabolites (MAPPD and DAPPD, respectively), i.e. de-toxified metabolites.

Mutagenicity
In COLIPA Submission III (2000) and the recent opinion of the SCCNFP (2002), the results of a number of genotoxicity studies were reviewed. More than 20 studies on the genotoxicity of p-phenylenediamine have been published in the toxicological literature. However, key studies reviewed in the SCCNFP opinion were investigations reported in the scientific literature, which were performed under a variety of conditions and/or did not correspond to GLP standards. In these tests, p-phenylenediamine was tested alone and/or in combination with hydrogen peroxide and/or couplers, the test products had unknown purities, auto-oxidation was not excluded, exposure times in the respective test systems exceeded by far the time of human contact with p-phenylenediamine-containing oxidative hair dyes, or combinations of p-phenylenediamine and couplers were tested that did not correspond to commercial products.
In these tests, when tested alone under *in vitro* conditions p-phenylenediamine resulted in contradictory, both positive and negative results. Positive results were found in the gene mutation test in bacteria (particularly in TA98 and TA 1538), in the gene mutation test in mammalian cells and in the chromosomal aberration test. *In vivo* micronucleus assays in rats and mice and an *in vivo* Comet assay did not confirm these positive *in vitro* findings.
In order to complete the safety dossier on p-phenylenediamine, it was decided to generate a new battery of genotoxicity studies under stringent conditions concerning test material and test protocols.

The earlier *in vitro* results were confirmed in the newly submitted *in vitro* genotoxicity assay performed according to OECD guidelines and with GLP compliance. In an *in vitro* gene mutation test in bacteria a positive result in strain TA98 after p-phenylenediamine treatment both with and without S9 was found. In contrast p-phenylenediamine exposure did not result in an

increase of the mutation frequency in an *in vitro* gene mutation assay in mammalian cells at the *hprt* locus. p-Phenylenediamine was positive in an *in vitro* micronucleus test.
Recently, two metabolites of p-phenylenediamine, N-monoacetyl para-phenylenediamine and N,N'-diacetyl para-phenylenediamine did not induce mutations in the gene mutation test in bacteria nor chromosome aberrations in human lymphocytes.
As was seen before in the older *in vivo* tests, also the newly submitted tests did not confirm these *in vitro* positive results. Both an *in vivo* micronucleus test and an *in vivo* unscheduled DNA synthesis test were negative.

In vitro in combination with an oxidising agent like hydrogen peroxide, p-phenylenediamine was also mutagenic in bacterial cells but not in mammalian cells and clastogenic in mammalian cells. Combination of p-phenylenediamine with an oxidising agent and a coupler, e.g. resorcinol, resulted in contradictory results in the gene mutation assay in bacteria whereas this combination was negative in the gene mutation test in mammalian cells and the chromosome aberration test. *In vivo* studies with the combinations have not been submitted.

Apparently, in the absence of oxidising agents and/or couplers the genotoxicity of p-phenylenediamine observed *in vitro* does not lead to genotoxic effects in experimental animals under appropriate test conditions and under this restriction p-phenylenediamine can be considered not genotoxic
To complete the safety dossier on p-phenylenediamine, additional *in vivo* genotoxicity studies with combinations of p-phenylenediamine and oxidising agents and couplers have to be performed.

Carcinogenicity
p-Phenylenediamine alone has not been demonstrated to be carcinogenic in experimental studies with rats or mice.
Hair dye formulations of p-phenylenediamine together with hydrogen peroxide have not been demonstrated to be carcinogenic in experimental studies after topical application to mice. The sensitivity of these studies may have been low as they did not respond to hair dye formulations containing known carcinogens. Thus, no conclusions with regard to carcinogenicity can be drawn from the studies.
Hair dye formulations of p-phenylenediamine together with hydrogen peroxide have been tested in three experimental studies after topical application to rats. The sensitivity of one of these studies may have been low as no response to hair dye formulations containing known carcinogens were observed. Thus, no conclusions with regard to carcinogenicity can be drawn from this study.
In two studies, mixtures of p-phenylenediamine and hydrogen peroxide or p-phenylenediamine and resorcinol and hydrogen peroxide were tested by topical application as well as by subcutaneous injection of rats. In both studies a statistical significant increase mammary gland tumours were found both after topical application as well as after subcutaneous injection. The studies have been criticized. However, it is not possible to disqualify the studies completely.
p-Phenylenediamine together with hydrogen peroxide may be carcinogenic in experimental studies with rats.

Safety assessment
A Common approach of the SCCP: From a 90 day study, a NOAEL of 4 mg/kg bw was obtained and is used as the basis for the safety evaluation. The highest cumulative penetration obtained in relevant studies on percutaneous absorption of PPD was 4.47 $\mu g/cm^2$. This leads to a margin of safety of 77.

B Toxicokinetic approach: In human volunteers (N=8), hair dyeing with a dark-shade, 2.0% [^{14}C]-PPD-containing hair dye produced a mean $AUC_{0\text{-}24h}$ of 0.66 μg_{eq} * hr / mL (mean value of individual AUCs). Comparison of the human plasma AUC with that of rats at the NOAEL of a subchronic oral toxicity study (4.0 mg/kg/day) yields a toxicokinetic-based safety margin of 16.3-fold. Given the AUC figures obtained from rats and humans the 4-fold factor for interspecies differences in toxicokinetics could be set to 1 which results in a remaining safety factor of 25 which was not achieved.

4. CONCLUSION

For the final safety assessment of PPD several aspects have to be taken into account:

- The SCCP considers PPD alone as being not genotoxic. But, positive findings from genotoxicity studies *in vivo/in vitro* of PPD in combination with couplers and /or hydrogen peroxide as well in a carcinogenicity study were reported.
- The generally accepted approach (MOS approach) according to the Notes of Guidance results in a MoS of 77. However, when toxicokinetic studies are considered, a minimum MoS of 25 can be set. A number of toxicokinetic studies were performed and the applicant proposed to base the safety on the comparison of AUCs (area under curve). In this approach, the AUC in rats following a peroral dosage of 4 mg/kg (corresponding to the NOAEL) was compared to the AUC in humans following application of a hair dye containing ^{14}C-labeled PPD. In this case a safety margin of 16.3 was obtained which is not considered sufficient by the SCCP.
- On the other hand, experimental evidence was provided that PPD is metabolised in the skin to acetylated (i.e. detoxified) derivatives and, furthermore, that presumably activation of PPD (formation of monoxygenated derivatives) does not occur.

The SCCP is of the opinion that the information submitted is insufficient to allow a final risk assessment to be carried out. Before any further consideration, additional data would be required on *in vivo* genotoxicity and/or carcinogenicity of PPD in combination with hydrogen peroxide and couplers (to simulate consumer exposure). Further information is needed in supporting the applicant's view that the MoS is sufficiently high.

There is an increasing use of hair dyes by young people and additional exposure to PPD-related substances from temporary tattoos and clothing textiles. PPD is an extreme sensitiser and the risk of allergy occurring in the consumer should be realised.

5. MINORITY OPINION

Not applicable

6. REFERENCES

Submission IV, July 2005

1. Ryle PR and Prentice DE. P-phenylenediamine (PPD). Expert opinion on carcinogenic potential. PreClinical safety Consultants Ltd. Muttenz, Switzerland, May 2005
2. Stanley LA, Skare JA, Doyle E, Powrie R, d'Angelo DD, Elcombe CR, 2005. Lack of evidence for metabolism of para-phenylenediamine by human cytochrome P 450 enzymes. *Toxicology* 210, 147-157
3. Powrie, R, Human hepatic metabolism of p-phenylenediamine: *in vitro* analysis. CXR Biosciences Ltd., Dundee, Scotland UK, Study No. CXR0228, 23 February 2005
4. Hueber-Becker F, Nohynek GJ, Meuling WJA, Benech-Kieffer F and Toutain H, 2004. Human systemic exposure to a [^{14}C]-paraphenylenediamine-containing oxidative hair dye and correlation with *in vitro* percutaneous absorption in human and pig skin. *Food Chemical Toxicology* 42, 1227-1236.
5. Meuling WJA. Dermal penetration of [^{14}C]-paraphenylenediamine after application to the hairy scalp of a PPD-containing permanent hair dye in male volunteers, TNO Zeist, The Netherlands, Study No. 4575, 7 January, 2003
6. Nohynek GJ, Skare JA, Meuling WJA, Hein DW, de Bie ATHJ, Toutain H, 2004. Urinary acetylated metabolites and N-acetyltransferase-2 genotype in human subjects treated with a para-phenylenediamine-containing oxidative hair dye. *Food and Chemical Toxicology* 45, 1885-1891.
7. Meuling WJA. Metabolic profiling of urinary p-phenylenediamine (PPD) metabolites and genotyping of male subjects exposed to a [^{14}C]-PPD-containing hair dye (2004), TNO, Zeist, The Netherlands, Study No. 5195, 31 March, 2004
8. Nohynek GJ, Duche D, Garrigue A, Meunier PA, Toutain H, Leclaire J, 2005. Under the skin: biotransformation of para-aminophenol and para-phenylenediamine in reconstructed human epidermis and human hepatocytes. *Toxicology Letters*, in print
9. Appleqvist T. Pharmacokinetics and mass balance of radioactivity in Sprague Dawley rats following single oral and dermal administration of [^{14}C]-PPD by oral gavage. CIT, Evreux, France, Study No. 26336 PAR, 3 January 2005
10. Appleqvist T. Pharmacokinetics and mass balance of radioactivity in Sprague Dawley rats following single administration of [^{14}C]-PPD by oral gavage. CIT, Evreux, France, Study No. 27160 PAR, 3 January 2005
11. SCCNFP, 2002. Opinion of the Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers concerning p-Phenylenediamine, COLIPA No. A7, adopted on February 27, 2002
12. SCCNFP, 2002. Opinion of the Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers concerning p-Phenylenediamine, COLIPA No. A7, adopted on February 27, 2002
13. SCCNFP, 2003. The SCCNFP's Notes of Guidance for the Testing of Cosmetic Ingredients and Their Safety Evaluation. 5th Revision. Adopted during the 25th plenary meeting of 20 October, 2003, p. 87. Available at: http://europa.eu.int/comm/health/ph_risk/committees /sccp/documents/out242_en.pdf
14. Renwick AG, Lazarus NR, 1998. Human variability and non-cancer risk assessment. An analysis of the default uncertainty factor. *Regulatory Toxicol. Pharmacol.* 27, 3-20, 1998.

References (SCCNFP/0129/99 of 27 February 2002)

13. Biodynamics Inc. (1977) A modified Segment II Teratology Study of Hair Dyes in Mice
16. Bracher, M. et al (1990) Studies on the Potential Mutagenicity of p-phenylenediamine in oxidative hair dye mixtures. Mutation Research 241, 313-23
19. Burnett CM, Lanman B, Giovacchini R, Wolcott G, Scala R, Keplinger M. Long-term toxicity studies on oxidation hair dyes. Fd Cosmet Toxicol 13: 353-357, 1975.
20. Burnett, C. et a] (1976) Teratology and Percutaneous Toxicity Studies on Hair Dyes. Journal of Toxicology and Environmental Health 1, 1027-1040
24. Burnett C et al (1981). Heritable translocation study on two hair dye formulations. Fundam. Appl Toxicol 1, 325-328
25. Burnett, C. et at (1982) The Effect of Dimethylsulfoxide on the Mutagenicity of the Hair Dye p-Phenylenediamine. Mutation Research 103, 1-4
26. Burnett CM, Goldenthal EI. Multigeneration reproduction and carcinogenicity studies in Sprague-Dawley rats exposed topically to oxidative hair-colouring formulations containing p-phenylenediamine and other aromatic amines. Fd Chem Toxicol 26 :467-474, 1988.
28. Chung, K.T. et al (1996) Effects of the nitro-group on the mutagenicity and toxicity of some benzamines. Environ. Mol. Mutagen. 27(1), 67-74
30. Crebelli R., Conti L., Carere A., Mutagenicity of commercial p-Phenylenediamine and of an oxidation mixture of p-Phenylenediamine and Resorcinol in *Salmonella typhimurium* TA98. Zito R. Fd. Cosmet. Toxicol. 19: 79-84 (1981).
42. Giles AL, Chung CW, Kommineni C. Dermal carcinogenicity study by mouse skin painting with 2,4-toluenediamine alone or in representative hair dye formulations. J Toxicol Environ Health 1: 433-440, 1976.
43. Goetz, N. et al (1988) Percutaneous absorption of p-Phenylenediamine during an actual hair dyeing procedure. Int J of Cosm Science 10, 63-73
46. Lochry E.A., Subchronic oral neurotoxicity study of H-18508 in rats. Haskell Laboratory for Toxicology and Industrial Medicine, Newark, Delaware. Report n° 854-91 (1992)
47. Hagiwara, A., Tamono, S., Shibata, M.A., Arai, M., Tsuda, H. Lack of modifying effects of p-phenylenediamine on induction of gamma-glutamyl traspeptidase-positive foci in a medium-term bioassay system using F344 rats. Toxicol Lett 52: 261-268, 1990.
49. Hausen B, Kaatz M, Jappe U, Stephan U, Heidbreder G. Henna/p-Phenylendiamin-Kontaktallergie Deutsches Arzteblatt. Jg 98. Heft 27. 6.7.2001
50. Holmberg, B., Kronevi, T., Ackevi, S., Ekner, A. Prövning av carcinogen aktivitet hos p-fenyllendiamin med peroral administrering på gravida möss (transplacentalförsök). Arbete och Hälsa 32: 1-44, 1983.
51. Holmberg, B., Kronevi, T., Ackevi, S., Ekner, A. Prövning av carcinogen aktivitet hos p-fenylendiamin med intraperitoneal injektion på nyfödda möss (neonataförsök. Arbete och Hälsa 33: 1-35, 1983.
52. Hossack, D. et al (1977) Examination of the Potential Mutagenicity of Hair Dye Constituents Using the Micronucleus Test. Experentia 33, 377
53. Imaida, K., Ishihara, Y., Nishio, O., Nakanishi, K., Ito, N. Carcinogenicity and toxicity tests on p-phenylenediamine in F344 rats. Toxicol Lett 16: 259-269, 1983.
58. Kalopissis, G. (1986) Toxicology and Hair Dyes. In : The Science of Hair Care, Zviak, C (ed.), Marcel Dekker, New York, 287-308
66. Le Coz C, Lefebre C, Keller F, Grosshans E. Allergic contact dermatitis caused by skin painting (pseudo tattooing) with black henna, a mixture of henna and p-phenylenediamine and its derivatives. Arch Dermatol 2000; 136: 1515-1517.
67. Lloyd, G. K. et al (1977) Assessment of the acute toxicity and potential Irritancy of hair dye constituents. Fd. Cosmet. Toxicol. 15, 607

71. Maouad, M. et al (1999) Significance-prevalence index number : A reinterpretation and enhancement of data from the North American Contact Dermatitis Group. J. Am. Acad. Dermatol. 41(4), 573-576
73. Maronpot R.R., Simkin M.B., Witschi L.H., Smith L.H., Cline J.M. Strain A mouse pulmonary tumor test results for chemicals previously tested in the National Cancer Institute carcinogenicity tests. JNCI 76: 1101-1112, 1986.
78. McFadden JP, Wakelin SH, Holloway DB, Basketter DA. Contact Dermatitis 39; 79-81,1998
79. Mitchell, A.D. et al (1988)Env. Mol. Mut. 1 (supl 13):37-101
86. National Cancer Institute. Bioassay of p-phenylenediamine dihydrochloride for possible carcinogenicity (Tech Rep Ser No 174), DHEW Publ No (NIH) 79-1730, 1979.
93. Re, T.A. et al (1981) The absence of teratogenic hazard potential of p-Phenylene diamine in Sprague-Dawley Rats. Fund Appl Toxicol 1, 421-425
96. Reprotox (1978) Prüfung auf embryotoxische Wirkung an der Maus : p-Phenylendiamin
97. Rojanapo W, Kupradinun P, Tepsuwan A, Chutimataewin S, Tanyakaset M. Carcinogenicity of an oxidation product of p-phenylenediamine. Carcinogenesis 7: 1997-2002, 1986.
98. Rojanapo W, Chutimataewin S, Kupradinun P, Tepsuwan A, Tanyakaset M, Satitnimankarn T, Sombooncharoen S. Carcinogenicity of hair dyes used in Thailand. Thai Cancer J 12: 43-56, 1986.
101. Sahoo B, Handa S, Penchallaiah K, Kumar B. Contact Dermatitis 43; 244, 2000
102. Sasaki, Y.F., et al (1999) The alkaline single cell gel electrophoresis assay with mouse multiple organs: results with 30 aromatic amines evaluated by the IARC and U.S. NTP Mutation Research 440,1-18
104. Segre, (1977) Rapport concernant les essais do toxicité aiguë chez la souris. Universita di Siena
108. Shahin, M.M. et al (1980) Studies on the mutagenicity of resorcinol and hydroxy-3-(pamino)anilino-6,N-[(p-amino)phenol]benzoquinone-monoimine-1,4 in Salmonella typhimurium. Mutation Research 78(3), 2113-18
112. Schnuch A, Geier J, Uter PJ et al. National rates and regional differences in sensitisation to allergens of the standard series. Contact Dermatitis 1997; 37: 200-209.
114. Spector, W. S, ed. (1956) Handbook of Toxicology, Volume 1. Acute toxicities of solids, liquids and gases to laboratory animals. W.B, Saunders Co., Philadelphia, 232
115. Stenbäck FG, Rowland JC, Russel LA. (1977) Noncarcinogenicity of Hair Dyes : Lifetime Percutaneous Applications in Mice and Rabbits. Fd. Cosmet. Toxicol. 15: 601-606, 1977.
119. Tosti A, Pazzaglia M, Betazzoni M. Contact allergy from temporary tattoos. Arch Dermatol 2000; 136: 1061-1062.
120. Toxicol Laboratories Limited, Herefordshire, England (1993). Paraphenylenediamine. 14-day oral (gavage) range-finding toxicity study in the rat. Report n° LRL/43/93
121. Toxicol Laboratories Limited, Herefordshire, England (1995). Paraphenylenediamine - 13-week oral (gavage) toxicity study in the rat. Report n° LRL/44/94 (1995)
125. Wakelin SH, Creamer D, Rycroft RJG, White IR, McFadden JP. Contact dermatitis from paraphenylenediamine used as a skin paint. Contact Dermatitis 1998; 39: 92-93.
126. Warbrick EV, Dearman RJ, Basketter DA, Kimber I. J Appl Toxicol 19; 255-260, 1999
129. Wolfram, L. J. et al (1985) Percutaneous Penetration of Hair Dyes. Arch Dermatol Res 277, 235-241

Additional references

A. Burnett C, Jacobs MM, Seppala A and Shubik P. Evaluation of the Toxicity and Carcinogenicity of Hair Dyes. J. Toxicol. Environ. Health 6: 247-257, 1980.
B. Goldenthal, E. Formulations 7401, 7402, 7403, 7404, 7405, 7406: Lifetime Toxicity / Carcinogenesis Study in Rats. IRDC Study No. 355-003 (a), 1979
C. Goldenthal EL. Lifetime Toxicity/Carcinogenesis Study in Rats, Report to Clairol Inc., International Research and Development Corporation, April 10, 1979.
D. WHO (1994) Assessing human health risks of chemicals: derivation of guidance value for health-based exposure limits. IPCS Environmental Health Criteria 170, WHO, Geneva
E WHO (1999) Principles for the assessment of risks to human health from exposure to chemicals. IPCS Environmental Health Criteria 210, WHO, Geneva
F. Sosted H, Johansen JD, Andersen KE and Menne T. Severe allergic hair dye reactions in 8 children. Contact Dermatitis 54: 87-91, 2006
G. Garrigue J-L, Ballantyne M, Kumaravel T, Lloyd M, Nohynek GJ, Kirkland D, Toutain H, (2006) *In vitro* genotoxicity of *para*-phenylenediamine and its *N*-monoacetyl or *N,N'*-diacetyl metabolites. Mutation Res., in press

References additional submission, December 2005

1. p-Phenylenediamine – single dose toxicity study by the oral route in the rat. MDS Pharma Services, L'Arbresle, France. Study No. AA28928, December 2005
2. OR10432 (p-phenylenediamine) – Local Lymph Node Assay. MDS Pharma, L'Arbresle, France. Study No. 413/600, May 2003
3. p-Phenylenediamine HCl: Reverse mutation assay in five histidine-requiring strains of Salmonella typhimurium. Covance Laboratories, Harrogate, UK. Study No. 413/125, September 2005
4. p-Phenylenediamine HCl: Mutation at the hprt locus of L5178Y Mouse Lymphoma Cells using the Microtitre Fluctuation Technique, Covance Laboratories, Harrogate, UK. Study No. 413/123, September 2005
5. p-Phenylenediamine HCl: Induction of micronuclei in cultured human peripheral blood lymphocytes. Covance Laboratories, Harrogate, UK. Study No. 413/122, December 2005
6. Micronucleus assay in bone marrow cells of the rat with p-phenylenendiamine. Audited draft report. Study Number 902401, RCC-CCR, Rossdorf, Germany, December 2005
7. *In vivo* unscheduled DNA synthesis in rat hepatocytes with p-phenylenendiamine. Draft report. Study Number 902402, RCC-CCR, Rossdorf, Germany, October 2005
8. p-Phenylenediamine – Embryotoxicity study by the oral route (gavage) in the rat (Segment II). Draft report, MDS Pharma, L'Arbresle, France. Study No. AA29083, December 2005
9. p-Phenylenediamine - Analytical method validation to determine test item concentrations in formulations. Draft report, MDS Pharma, L'Arbresle, France. Study No. AA29403, November 2005

7. ACKNOWLEDGEMENTS

Members of the working group are acknowledged for their valuable contribution to this opinion. The members of the working group are:

Dr. C. Chambers
Prof. R. Dubakiene
Prof. V. Kapoulas
Prof. C. Lidén
Prof. J.-P. Marty
Prof. T. Platzek (chairman & rapporteur)
Dr. S.C. Rastogi
Prof. T. Sanner
Dr. J. van Engelen
Dr. I.R. White

External expert

Dr. M.-L. Binderup	Danish Institute for Food and Veterinary Research, Denmark
Dr. H. Norppa,	Institute of Occupational Health, Finland
Dr. K. Peltonen,	EVIRA, Finland
Dr. J. van Benthem	RIVM, the Netherlands