Scientific Committee on Consumer Safety

SCCS

OPINION ON

p-Phenylenediamine

COLIPA n° A7

The SCCS adopted this opinion at its 15th plenary meeting

Of 26 – 27 June 2012

About the Scientific Committees

Three independent non-food Scientific Committees provide the Commission with the scientific advice it needs when preparing policy and proposals relating to consumer safety, public health and the environment. The Committees also draw the Commission's attention to the new or emerging problems which may pose an actual or potential threat.
They are: the Scientific Committee on Consumer Safety (SCCS), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and are made up of external experts.

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

SCCS

The Committee shall provide opinions on questions concerning all types of health and safety risks (notably chemical, biological, mechanical and other physical risks) of non-food consumer products (for example: cosmetic products and their ingredients, toys, textiles, clothing, personal care and household products such as detergents, etc.) and services (for example: tattooing, artificial sun tanning, etc.).

Scientific Committee members

Contact
European Commission
Health & Consumers
Directorate D: Health Systems and Products
Unit D3 - Risk Assessment
Office: B232 B-1049 Brussels
Sanco-SCCS-Secretariat@ec.europa.eu

ISSN 1831-4767 ISBN 978-92-79-30767-6
Doi:10.2772/8451 ND-AQ-12-017-EN-N

ACKNOWLEDGMENTS

Prof. J. Angerer
Dr. C. Chambers
Dr. E. Nielsen
Dr. W. Lilienblum (associated scientific advisor)
Prof. T. Platzek (chairman, rapporteur)
Dr. S.C. Rastogi
Dr. C. Rousselle
Prof. T. Sanner
Dr. J. van Benthem
Prof. M.P. Vinardell
Dr. I.R. White

External experts

Dr. Mona-Lise Binderup National Food Institute, Denmark

Keywords: SCCS, scientific opinion, hair dye, A7, p-phenylenediamine, PPD, directive 76/768/ECC, CAS 106-50-3, EC 203-404-7

Opinion to be cited as: SCCS (Scientific Committee on Consumer Safety), Opinion on p-phenylenediamine, 26-27 June 2012

This opinion has been subject to a commenting period of four weeks after its initial publication. Comments received during this time have been considered by the SCCS and discussed in the subsequent plenary meeting. Where appropriate, the text of the relevant sections of the opinion has been modified or explanations have been added. In the cases where the SCCS after consideration and discussion of the comments, has decided to maintain its initial views, the opinion (or the section concerned) has remained unchanged.
Revised opinions carry the date of revision.

TABLE OF CONTENTS

1. BACKGROUND

Submission I on *p*-Phenylenediamine (PPD) was submitted in December 1979 by COLIPA[1] according to COLIPA.

Submission II on *p*-Phenylenediamine was submitted in March 1983 by COLIPA according to COLIPA.

Submission III on *p*-Phenylenediamine was submitted by COLIPA in June 2000 according to COLIPA.

Submission IV on *p*-Phenylenediamine was submitted by COLIPA in July 2005 according to COLIPA.

The Scientific Committee on Cosmetic Products expressed its opinion (SCCP/0989/06) with the following conclusions:

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 and 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 bw (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 are 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.

The substance is currently regulated in 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, with a concentration limit of 2.0%.

Submission V on *p*-Phenylenediamine was submitted by COLIPA in July 2011. This submission summarizes the results and conclusions obtained in recent studies and reports, in which the substance was used in oxidative hair colouring products containing at on-head concentration of up to 2.0%.

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association

2. TERMS OF REFERENCE

1. *Does SCCS 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. *And/or does the SCCS recommend any further restrictions with regard to the use of p-Phenylenediamine in any 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 dihydrochloride and sulfate (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

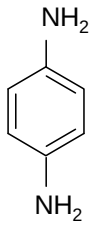
3.1.1.3. Trade names and abbreviations

PPD
CI 76 060
COLIPA n° A7

3.1.1.4. CAS / EC number

	free base	dihydrochloride	sulfate
CAS:	106-50-3	624-18-0	541-70-8; 25723-55-4
EC:	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 g/mol (base)
181.07 g/mol (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:

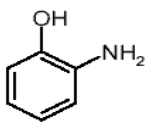
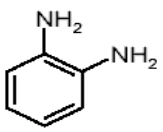
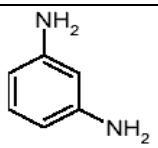
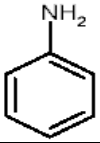
03629102 (in April 1993), 976070 (in October 1997), 1269 (in March 2004), 2G100 (in March 2004), 2L389 (in March 2004)
The analytical study of p-phenylenediamine (dihydrochloride) was performed on one batch: 000 D002 (in April 2005)

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 was 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: /
Loss on drying: /
Ash: < 0.1 g/100 g
Solvents: < 100 ppm
Hg, Sb, As: each < 5 ppm (mg/kg bw)
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, submission 2005, the solubility in water is at least 1%.

3.1.7. Partition coefficient (Log P_{ow})

Log P_{ow}: - 0.31 (calculated)

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 properties

- No data on stability in test solutions and in typical hair dye formulations were submitted.
- Log Pow: 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.

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.

The SCCS is aware that also the sulfate and dihydrochloride salts of p-phenylenediamine are used in oxidative hair dye formulations.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

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 bw died within 3 h. The animal treated with 50 mg/kg bw showed clinical signs (lacrimation, swelling of conjunctivae, gait, tremor, subdued behaviour and/or piloerection). At 25 mg/kg bw only orange traces in the bedding were seen probably due to coloured urine.

Ref.: 1 (suppl. data 2005)

Acute toxicity has been investigated following oral, subcutaneous, intraperitoneal and topical application in a variety of species. The LD50 following oral administration was 80-100 mg/kg bw in the rat, 290 mg/kg bw in mice, 250 mg/kg bw in rabbit and 100 mg/kg bw in cats.

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

The intraperitoneal and topical LD50 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 p-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

Human Poisoning

Accidental and intentional hair dye ingestion in East Africa and India is not uncommon. The main component is p-phenylenediamine which is known to cause angioneurotic oedema, rhabdomyolysis and renal failure. Also fatal cases of myocarditis were reported.

Additional Ref.: J, K, L

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 SCCP/0989/06

A 2.5% aqueous solution of p-phenylenediamine 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 SCCP/0989/06

A 2.5% aqueous solution of p-phenylenediamine 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

Animal data

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

Local lymph node assay (LLNA), *submission IV, additional data December 2005*

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% trichloroacetic 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 (suppl. data, 12/2005)

Human data

The list below is not intended to be an exhaustive review of literature but is indicative of the problem:

p-Phenylenediamine (PPD) is a known extreme skin sensitiser and included in the European Baseline Series for diagnostic patch testing of eczema patients, apart from in Germany, where the risk of active sensitisation from routine diagnostic patch testing with PPD is considered to be unacceptably high.

Additional Ref.: R

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

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

Standard patch tests in more than 36000 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 thiomersal. There was considerable regional difference in the sensitisation rates when different regions of Germany were compared.

Ref.: 112

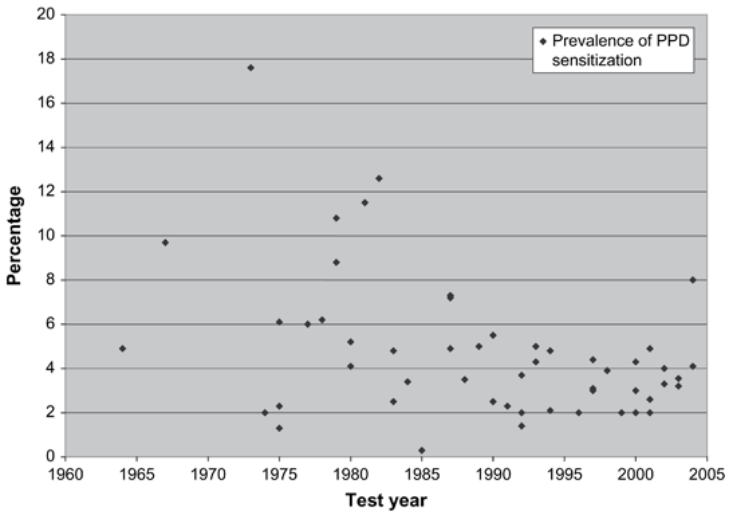
The North American Contact Dermatitis Group reported in 1999 that in spite of its potential allergenicity, the PPD SPIN value (Significance Prevalence index Number), which corresponds to the quantitative measure of the relative clinical importance of contact allergens in the population, remained relatively 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

p-Phenylenediamine (PPD) is a frequent and important component of permanent hair dye products; exposure to it may cause allergic contact sensitization, acute dermatitis, and severe facial oedema. Affected individuals are at risk of clinically severe reactions upon hair dyeing. A warning concerning the risk and severity of allergic reactions appears on products containing PPD (and similar phenylenediamines).

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.

A recent review of published literature containing PPD patch test data from dermatitis patients and individuals in the general population was undertaken to estimate the median prevalence and the weighted average of PPD sensitization and thereby assess the burden of PPD-containing hair care products on health. Literature was examined using PubMed–MEDLINE, Biosis, and Science Citation Index. The median prevalence among dermatitis patients was 4% in Europe. In Europe, a decrease in the 1970s was replaced by a plateau with steady, high prevalence ranging between 2% and 6%.



The above chart represents data collected from many centres in a variety of European countries over several decades.

Additional Ref.: Q

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

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.

Additional Ref.: F

Immediate reactions to hair dyes

Reports have described immediate hypersensitivity reactions, including anaphylaxis due to PPD in hair dyes.

Ref.: 101
Additional Ref.: X

The immediate reactions reported consist of localised bumps on the head to widespread urticarial lesions and angioedema and respiratory distress. The following are illustrative:

- The case report of Fukunaga et al. describes a woman who experienced "episodes of itchy bumps on her head" and hands 10-30 minutes after applying the dye, which contained para-phenylenediamine. The bumps disappeared 3 h later. After her most recent use of the dye, the itchy bumps had spread over her entire body and she had developed nausea, vomiting and difficulty in breathing, accompanied by a cold sweat ...". Investigations provided strong evidence that this woman was Type 1 sensitive to p-phenylenediamine and passive transfer was achieved.

  Additional Ref.: S

- An important case report is that of Goldberg et al. (1987):
  A woman developed swollen eyes and itchy hands within ten minutes of application of a hair dye. Some months later, she used a different product and again developed swollen eyes and also dysphonia and palpitations. Two months later, a third hair dye caused similar symptoms. An antihistamine was administered. A few years later the original dye was used again. Within 10 minutes she experienced scalp burning, blurry vision, itching of the palms etc. She developed angioedema of the face and hands, dyspnoea and lost consciousness. Antihistamine was administered and in the local casualty department she was observed to have angioedema of her face with a maculopapular rash.
  Investigations produced an immediate (within 20 minutes) wheal and flare reaction to the hair dye mixture and further investigations showed that an oxidation product of p-phenylenediamine (Bandrowski's base) to be the prime allergen. Passive transfer was achieved confirming the immediate hypersensitivity.

  Ref.: T

- In the UK, a case report (Mavroleon et al. 1998)
  It describes a woman with a history suggestive but not diagnostic of an immediate reaction to hair dye because of a confounding factor (a drug). She was challenged in hospital by dying a "small part" of her hair. No effect was observed. The investigators then dyed the rest of her hair and after 20 minutes she reported palpitations and chest tightness; she became faint and flushed. She developed a generalised urticarial rash.

  Ref.: U

- The case report of Pasche-Koo et al describes:
  A woman who developed extensive urticarial lesions starting on her scalp and face followed by gastrointestinal symptoms and loss of consciousness, after 30 minutes of having her hair dyed. During the previous 3 years, she had experienced contact urticaria localized to the scalp and neck and in the previous 2 years the urticaria and asthma after having her hair dyed. Investigations produced no reaction on open testing but on prick testing there was good evidence that the oxidation product of p-toluenediamine (PTD) was responsible for the reaction. "*PTD and PPD are closely related compounds, and their oxidation products are chemically close ..... Immediate reactions to hair-dye constituents, although rare, may be life-threatening ...*"
  Ref.: V

Death as a result of anaphylactic reactions to PPD have been suspected.

- The case report of Belton and Chira (1997) describes a 68 year old woman who "complained to her son that she felt short of breath soon after applying a hair dye preparation .." Two years before her death, after using another hair dye she "showed signs of a diffuse erythematous rash on both shoulders and legs and experienced severe shortness of breath and crushing chest pain". On this occasion it was shown that she had had a myocardial infarction which was attributed to "combined stress of anaphylaxis and epinephrine therapy".
  Ref.: W

Comment
The frequency of immediate hypersensitivity reactions to PPD in hair dyes is unknown but severe reactions appear to be rare in comparison to the volume of PPD containing hair dyes used.

3.3.4. Dermal / percutaneous absorption

Taken from SCCP/0989/06

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/cm² of 1.3% PPD and other dyes in the presence of developer, but the absence of hair
b) 100 mg/cm² of 1.3% PPD and other dyes in the presence of developer and hair
c) 100 mg/cm² of 2.7% PPD, but no other dyes, developer or hair
d) 20 mg/cm² of 2.7% PPD, but no other dyes, developer or hair
e) 100 mg/cm² 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/cm² 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/cm² 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/cm². 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/cm² in the *in vitro* studies and 4.5 µg/cm² in 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 as 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/cm², based on an estimated scalp area of 700 cm².

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. (ref.: 14, 15).

3.3.5. Repeated dose toxicity

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

Taken from SCCP/0989/06

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 bw/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 10 mg/kg bw/day or greater (increased alanine and/or aspartate aminotransferase, creatinine phosphokinase and lactate dehydrogenase (LDH) levels). At 5 mg/kg bw only LDH was changed.
- 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 NOEL was < 5 mg/kg bw/day.

Ref.: 120

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

Taken from SCCP/0989/06

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 408 (1981). PPD was administered by gavage at corresponding dose levels of 2, 4, 8 and 16 mg/kg bw/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 bw/day and 16 mg/kg bw/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 NOEL of PPD was established at 4 mg/kg bw/day by the applicant and considered as NOAEL by the SCCNFP and SCCP.

Ref.: 121

External expert review (18, subm V)

In the expert review, it was stated that the liver and kidney weight increases at 8 and 16 mg/kg bw/d are the only changes in this study which were considered to be related to treatment with PPD. Such organ weight changes without associated clinical chemistry

alterations and without any corresponding histopathological changes were considered to be adaptive in nature and non-adverse. The external expert therefore concluded the following for this study:

- The no observed effect level (NOEL) is 4 mg/kg bw/d
- The no observed adverse effect level (NOAEL) is 16 mg/kg bw/d

This conclusion confirmed the NOEL as cited in the original report. It also expanded this original conclusion to include a judgement on adverse findings and defined the NOAEL for the study.

Comment of the SCCS
The SCCS agrees to this judgement of kidney and liver effects observed. However, also the observed myodegenerative effects need to be considered. It is well known that PPD and some substituted derivatives can induce myotoxicity (e.g. even after 3 days of administration of 36.2 mg/kg bw, see Additional References H and I: Munday et al. 1990; Munday, Manns 1999). This myodegeneration in the skeletal muscle was also noted in the 28-day study in 3 females given 40 mg/kg bw/d (Ref. 120). Minimal myodegeneration on skeletal muscle was noted in the 90-day study on 1 male and 1 female of the high dose group (16 mg/kg bw). Although this was also observed in the respective control group a relation to PPD treatment cannot be excluded. SCCS sets the NOAEL at 8 mg/kg bw.

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 bw/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 13 weeks 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 bw/d for both sexes; the NOAEL was 16 mg/kg bw/d.

Ref.: Z

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 bw 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 SCCP/0989/06

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*

3.3.6.1.1 Genotoxicity tests with p-Phenylenediamine alone

Bacterial gene mutation test
(taken from SCCNFP/0129/99, adopted in 2002, modified)

p-Phenylenediamine has been tested for mutagenicity in *Salmonella typhimurium* strains TA98 and TA1538 in the presence of S9-mix. 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 was not genotoxic (mutagenic) in this gene mutation tests in bacteria.

Ref.: 25

Comment
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 data are from a publication in the open literature. The test was not conducted in compliance with GLP or OECD guidelines or according to present standards. The test has only limited value and can at most be used for confirmation purposes.

Bacterial gene mutation test
(taken from SCCNFP/0129/99, adopted in 2002, modified)

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 in strain TA98 of *Salmonella typhimurium* in the presence and absence of S9-mix obtained from rat-livers treated with Aroclor 1254. The concentrations tested ranged from 0 – 2000 µg/plate. Three replicate plates in 2 separate experiments were evaluated. In the first experiment the plate incorporation method was used, in the second experiment with purified p-phenylenediamine only the microtitre fluctuation method.

Results
A biologically relevant and dose dependent increase in the number of revertant colonies compared to the control value was observed for the commercial samples of p-phenylenediamine in the presence of S9-mix, but not in the absence of S9-mix. No biologically relevant increase in revertant colonies was found for in purified p-phenylenediamine and for commercial resorcinol both without and with S9-mix.

Conclusion
Under the experimental conditions used commercial samples of analytical grade p-phenylenediamine were genotoxic (mutagenic) in this gene mutation tests in bacteria whereas purified p-phenylenediamine was not mutagenic.

Ref.: 30

Comment
The data are from a publication in the open literature. The test was not conducted in compliance with GLP or OECD guidelines. The test has only limited value and can at most be used for confirmation purposes.

Bacterial gene mutation test
(taken from SCCNFP/0129/99, adopted in 2002, modified)

p-Phenylenediamine was tested in the gene mutation test in *Salmonella typhimurium* strains TA98 and TA100 and the nitroreductase-deficient strains TA98NR and TA100NR in the presence and absence of S9-mix. The pre-incubation procedure was used. Concentrations of 1 – 3000 µg/plate were tested. S9-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-mix; in TA100NR in the absence of S9-mix.

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

Ref.: 28

Comment
The data are from a publication in the open literature. The test was not conducted in compliance with GLP or OECD guidelines nor according to present standards. The test has only limited value and can at most be used for confirmation purposes.

Bacterial gene mutation assay, submission III

Guideline:	OECD 471
Species/strain:	*Salmonella typhimurium* TA98, TA100, TA102, TA1535, and TA1537
Replicates:	3 replicates in 2 individual experiments

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-mix
	experiment 2:	156.3 - 5000 µg/plate both without and with S9-mix
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-mix
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. Concentrations were chosen after a toxicity range-finder experiment with strain TA100 in the absence and presence of S9-mix using concentrations up to 5000 µg/plate. Since p-phenylenediamine is an oxidizable 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-mix. In experiment 2, toxicity was found in the plate incorporation treatments of TA98 with S9-mix at the highest dose. Using the pre-incubation method toxicity was found for TA102 at 2500 and 5000 µg/plate with S9-mix and at 5000 µg/plate without S9-mix.
In experiment 1, statistically significant increases were found in TA100 without S9-mix 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 a positive result was found in strain TA98 in the presence of S9-mix 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
Therefore, under the experimental conditions used p-phenylenediamine was genotoxic (mutagenic) in this gene mutation tests in bacteria.

Ref.: 3 (suppl. data, 12/2005)

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

p-Phenylenediamine was reported to be positive in the *in vitro* L5178Y mouse lymphoma assay with and without metabolic activation. Without S9-mix, 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-mix 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

Comment
The data are from a publication in the open literature. The test was not conducted in compliance with GLP or OECD guidelines nor according to present standards. The test has only limited value and can at most be used for confirmation purposes.

In vitro Gene Mutation Assay (_hprt_ locus), submission III

Guideline:	OECD 476	
Species/strain:	mouse lymphoma L5178Y cells	
Replicates:	duplicate cultures 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-mix 25 – 900 µg/ml with S9-mix
	experiment 2:	2.5 – 60 µg/ml without S9-mix 25 – 1000 µg/ml with S9-mix
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-mix 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-mix and in the second experiment in the absence and presence of S9-mix 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 mutant frequency at the *hprt*-locus of mouse lymphoma cells was not observed. The only statistically significant increase in the mutant frequency (experiment 2 with S9-mix intermediate dose of 100 µg/ml) was not reproducible and therefore considered not biologically relevant.

Conclusion
Under the experimental conditions used p-phenylenediamine was not mutagenic in this *in vitro* gene mutation assay with mammalian cells.

Ref.: 4 (suppl. data, 12/2005)

Chromosome aberration test
(taken from SCCNFP/0129/99, adopted in 2002, modified)

p-Phenylenediamine was tested in a chromosome aberration using CHO-K1 cells. These cells were exposed in the absence of S9-mix 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 was genotoxic (clastogenic) in this chromosomal aberration test.

Ref.: 28

Comment
The data are from a publication in the open literature. The test was not conducted in compliance with GLP or OECD guidelines nor according to present standards. The test has only limited value and can at most be used for confirmation purposes.

In vitro micronucleus test, submission III

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	
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-mix 500, 900 and 1600 µg/ml with S9-mix
	experiment 2:	50, 100 and 125 µg/ml without S9-mix 400, 1400 and 2000 µg/ml with S9-mix
Treatment	experiment 1:	24 h PHA, 20 h treatment and 28 h recovery without S9-mix 24 h PHA, 3 h treatment and 45 h recovery with S9-mix
	experiment 2:	48 h PHA, 20 h treatment and 28 h recovery without S9-mix 48 h PHA, 3 h treatment and 45 h recovery with S9-mix
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 oxidizable compound it is was stored and diluted under nitrogen atmosphere. Test concentrations were based on the result of a range finding toxicity study. Toxicity was determined by measuring the reduction in replication index (RI). Treatment periods were 24 h without S9 and 3 h with S9-mix. 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. In every separate experiment various dilutions of p-phenylenediamine were tested. However, only 3 concentrations were analyzed. The top concentration for analysis was to be the one with at least 50 - 60% reduction in cytotoxicity. The lower concentrations 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-mix 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 cells with 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-mix fell within this range this increase was not considered biologically relevant.
In experiment 1 with S9-mix as well as in experiment 2 both without and with S9-mix a more or less dose dependent increase in micronucleated bi-nucleated cells was observed.

Conclusion
Under the experimental conditions used, p-phenylenediamine was genotoxic (clastogenic and/or aneugenic) in human lymphocytes *in vitro*.

Ref.: 5 (suppl. data, 12/2005)

3.3.6.1.2 Genotoxicity 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.
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-mix experiment 2: 156.3 - 5000 µg/plate without S9-mix
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 concentration in one

experiment was neither concentration-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 described in a publication, the raw data were not available. Moreover, the results from the top three concentrations only were reported. The test has only limited value.
However, although it was unclear whether the test was performed according an OECD guideline, the batch was not reported and the GLP status was not 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 to 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
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-mix DAPPD: experiment 1: 480, 600 and 700 µg/ml without S9-mix experiment 2: 384, 480 and 690 µg/ml without S9-mix
Treatment	experiment 1: 24h PHA, 20h treatment and 28h recovery without S9-mix experiment 2: 48h PHA, 20h treatment and 28h recovery without S9-mix
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. 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 concentration 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 concentration required by the OECD guideline (10 mM), was not toxic and induced approximately 4% reduction in RI. In the first experiment the highest concentration 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 concentration, 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 was not genotoxic (clastogenic and/or aneugenic) in human lymphocytes *in vitro*.

Ref.: G

Comment
Since this *in vitro* micronucleus test was described in a publication and the raw data were not available, the test has only limited value.
However, although it was unclear whether the test was performed according to an OECD guideline, the batch was not reported and the GLP status is not 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.

3.3.6.1.3 Genotoxicity tests with a reaction product of p-phenylenediamine and resorcinol in the presence of hydrogen peroxide

Bacterial gene mutation test
(taken from SCCNFP/0129/99, adopted in 2002, modified)

A reaction product obtained from a reaction between purified p-phenylenediamine and resorcinol in the presence of hydrogen peroxide 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 S9-mix obtained from rat livers treated with Aroclor 1254. The concentrations tested ranged from 0 - 2000 µg/plate. Three replicate plates in 2 separate experiments were evaluated. Both the plate incorporation method and the microtitre fluctuation method was used.

Results
A biologically relevant and dose dependent increase in the number of revertants compared to the value of the control was observed in the presence of S9-mix, but not in the absence of S9-mix.

Conclusion
Under the conditions of the tests the reaction product of resorcinol and p-phenylenediamine was genotoxic (mutagenic) in this gene mutation tests in bacteria.

Ref.: 30

Comment
The data are from a publication in the open literature. The test was not conducted in compliance with GLP or OECD guidelines. The test has only limited value and can at most be used for confirmation purposes.

Bacterial gene mutation test
(taken from SCCNFP/0129/99, adopted in 2002, modified)

A compound formed as a reaction product between resorcinol (pure commercial product from Hoechst) and p-phenylenediamine, hydroxyl-3-(p-amino)aniline-6,N-[(p-amino)phenol]-benzoquinonemonoimine-1,4, 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 1254 treated rat liver microsomes.

Five *Salmonella typhimurium* strains, TA98, TA100, TA 1535, TA1537, TA1538, were used. The concentrations ranged from 5 to 1000 μg/plate in DMSO in two independent experiments. The reaction product tested was synthesised and purified by re-crystallisation, and analysed by nuclear magnetic resonance spectroscopy (^{1}H NMR).

Results
Resorcinol as well as the reaction product did not induce a biologically relevant increase in the number of revertants in any of the 5 *Salmonella* strains.

Conclusion
Under the conditions of the tests the oxidation product of resorcinol and p-phenylenediamine was not genotoxic (mutagenic) in this gene mutation tests in bacteria.
Ref.: 108

Comment
The data are from a publication in the open literature. The test was not conducted in compliance with GLP or OECD guidelines.
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.

3.3.6.1.4 Genotoxicity tests with p-phenylenediamine and hydrogen peroxide without or with resorcinol

Bacterial gene mutation test
(taken from SCCNFP/0129/99, adopted in 2002, modified)

Guideline:	/
Species/strain:	*Salmonella typhimurium* TA98
Replicates:	triplicate cultures
Test substance:	mixture of 55 mM p-phenylenediamine dihydrochloride and 3% H_2O_2 without or with 66 mM resorcinol
Batch:	/
Purity:	/
Vehicle:	1% ammonia
Concentrations:	0.028, 0.055, 0.138, 0.275, 0.55, 1.375, 2.75 and 5.5 μM PPD/plate with S9-mix
Treatment:	standard plate incorporation method
GLP:	/

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 procedures were assayed in the gene mutation test with bacteria. *Salmonella typhimurium* strain TA98 was exposed in the presence of S9-mix obtained from rat-livers treated with Aroclor 1254. The mixture has been evaluated after 30 min of pre-incubation at 37 °C. The plate incorporation method was used. Negative and positive controls were included.

Results
In the presence of S9-mix, a biologically relevant and dose dependent increase in the number of revertants compared to the value of the control was found for the mixture of p-phenylenediamine and H_2O_2. The positivity is probably due to the formation of Bandrowski's bases which could be demonstrated with thin layer chromatography. However, a biologically relevant increase in the number of revertants was not found for the mixture of p-phenylenediamine with resorcinol and H_2O_2.

Conclusion
Under the conditions of the tests p-phenylenediamine in a mixture with H_2O_2 was genotoxic (mutagenic) in this gene mutation tests in bacteria; the mixture of p-phenylenediamine with resorcinol and H_2O_2 was not genotoxic (mutagenic).

Ref.: 16

Comment
The data are from a publication in the open literature. The test was not conducted in compliance with GLP or OECD guidelines.

In vitro gene mutation test in mammalian cells (_tk_ locus)
(taken from SCCNFP/0129/99, adopted in 2002, modified)

Guideline:	/
Species/strain:	mouse lymphoma L5178Y cell line
Replicates:	duplicate cultures
Test substance:	mixture of 55 mM p-phenylenediamine dihydrochloride and 3% H_2O_2 without or with 66 mM resorcinol
Batch:	/
Purity:	/
Vehicle:	1% ammonia
Concentrations:	0.02, 0.061, 0.184 and 0.552 μM PPD/ml with S9-mix
Treatment:	2 h treatment with S9-mix, expression period 7-9 days and a selection period of 14 days.
GLP:	/

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 procedures were assayed in the mammalian gene mutation system using mouse lymphoma L5178Y cells. A fluctuation protocol both in the absence and presence of S9 metabolic activation was used. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. The mixture has been evaluated after 30 min of pre-incubation at 37 °C. Cells were treated for 2 h both without and with S9-mix followed by an expression period of 7-9 days to fix the DNA damage into a stable mutation and a selection period of 14 days. 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 included.

Results
A biologically significant increase in the mutant frequency was not observed for both the mixture of p-phenylenediamine and H_2O_2 alone nor in combination with resorcinol and H_2O_2.

Conclusion
Under the conditions of the tests p-phenylenediamine in a mixture with H_2O_2 or in a mixture with resorcinol and H_2O_2 is not mutagenic in this gene mutation test in mammalian cells.

Ref.: 16

Comment
The data are from a publication in the open literature. The test was not conducted in compliance with GLP or OECD guidelines.

In vitro chromosome aberration test
(taken from SCCNFP/0129/99, adopted in 2002, modified)

Guideline: /

Cells:	Human lymphocytes of one male and one female donor)
Replicates:	duplicate cultures
Test substance:	mixture of 55 mM p-phenylenediamine dihydrochloride and 3% H_2O_2 without or with 66 mM resorcinol
Batch:	/
Purity:	/
Vehicle:	1% ammonia
Concentrations:	0.138, 0.276 and 0.552 μM PPD/ml without and with S9-mix
Treatment:	3 h without and with S9-mix; harvest time 28 h after start of treatment.
GLP:	/

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 procedures were assayed in the chromosomal aberration test with human lymphocytes. Whole blood cultures of 3 donors, one male and one female, were established. The mixture has been evaluated after 30 min of pre-incubation at 37 °C. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system.
Treatment periods were 3 h without and with S9-mix. Harvest times were 28 hours after the start of treatment. One h prior to harvest culture was in the presence of colchicine (at a final concentration of 1 µg/ml). Toxicity was determined by measuring the reduction in mitotic indices. Negative and positive controls were included.

Results
Both without and with S9-mix, treatment of human lymphocytes with p-phenylenediamine resulted in a concentration dependent increase when p-phenylenediamine was mixed with H_2O_2. On the other hand, a biologically relevant increase in the number of cells with chromosomal aberrations was not observed for the combination with resorcinol and H_2O_2 both without and with S9-mix. In the latter experiment with S9-mix an occasional statistical significant increase in the number of cells with chromosome aberrations was found for the mid concentration. However, as no concentration dependency was found and the increase occurred only in one culture, this increase was considered not biologically relevant.

Conclusion
Under the experimental conditions used, p-phenylenediamine in a mixture with H_2O_2 was genotoxic (clastogenic) for human lymphocytes but not in a mixture with resorcinol and H_2O_2.

Ref.: 16

Comment
The data are from a publication in the open literature. The test was not conducted in compliance with GLP or OECD guidelines.

3.3.6.1.5 Genotoxicity tests with Trimer A007-A017-A007 (reaction product of p-phenylenediamine and 1-naphthol)

Bacterial Reverse Mutation Test

Guideline:	OECD 471 (1997)
Species/strain:	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA102
Replicates:	triplicates in a single experiment
Test substance:	A007-A017-A007
Solvent:	DMSO
Batch:	GF814775
Purity:	98% (HPLC, area at 254 nm)

Concentrations:	3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate without and with S9-mix
Treatment:	direct plate incorporation with 72 h incubation without and with S9-mix
GLP:	In compliance
Date:	January 2009 - October 2009

The reaction product of p-phenylenediamine and 1-naphthol A007-A017-A007, dissolved in DMSO, was tested in bacterial tester strains of *Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537, in a single experiment both in the presence and in the absence of a metabolic activation system. Each concentration and the controls were tested in triplicate. Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system.
A pre-experiment for toxicity was performed with all strains both without and with S9-mix to select the concentrations for the main experiment. Toxicity was evaluated for 8 concentrations up to the prescribed maximum concentration of 5000 µg/plate on the basis of a reduction in the number of revertant colonies and/or clearing of the bacterial background lawn. The pre-experiment was performed as a plate incorporation assay. Since in this pre-experiment evaluable plates were obtained for five concentrations or more in all strains used, the pre-experiment is reported as the main experiment. Since a positive response was obtained in this experiment a second experiment was not performed.
Appropriate reference mutagens were used as positive controls and showed distinct increases in induced revertant colonies. Concurrent vehicle controls were performed as negative controls, and their mean numbers of revertant colonies all fell within acceptable ranges.

Results
The plates incubated with A007-A017-A007 showed normal background growth up to 5000 µg/plate with and without S9-mix in all strains used. Minor toxic effects, evident as a reduction in the number of revertants, occurred in the absence of metabolic activation in strain TA1535 at 2500 and 5000 µg/plate and in strain TA1537 at 5000 µg/plate.
A dose dependent increase in revertant colony numbers was observed following treatment with A007-A017-A007 in strains TA98 and TA1537 in the presence of S9-mix. A biologically relevant increase in revertant colonies was not observed in strain TA98 and TA1537 in the absence of S9-mix nor in any of the other strains used neither in the absence nor in the presence of S9-mix.
Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant colonies.

Conclusion
Under the experimental conditions used A007-A017-A007 was genotoxic (mutagenic) in this gene mutation tests in bacteria.

Additional Ref.: M

In vitro gene mutation assay (*hprt* locus)

Guideline:	OECD 476 (1997)	
Cells:	Chinese hamster V79 cells	
Replicates:	duplicate cultures in 2 independent experiments	
Test substance:	A007-A017-A007 (WR A021232)	
Solvent:	DMSO	
Batch:	GF814775	
Purity:	98% (HPLC, area at 254 nm)	
Concentrations:	experiment I:	0.125, 0.25, 0.5, 1, 2 and 2.5 µg/ml without S9-mix 1.25, 2.5, 5, 10, 15 and 20 µg/ml with S9-mix
	experiment II:	1, 1.5, 1.75, 2, 2.3 and 2.5 µg/ml without S9-mix 5, 10, 12.5, 15, 17.5 and 20 µg/ml with S9-mix

Treatment:	4 h treatment with and without S9-mix, expression period 7 days and a selection period of 8 days.
GLP:	In compliance
Date:	December 2008 - October 2009

The reaction product of p-phenylenediamine and 1-naphthol A007-A017-A007, dissolved in DMSO, was assayed for mutations at the *hprt* locus of Chinese hamster V79 cells both in the absence and presence of metabolic activation. Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. The study was performed in two independent experiments, using duplicate cultures and identical experimental procedures.
Test concentrations were based on the results of a pre-test on toxicity. Toxicity of A007-A017-A007 is indicated by a reduction of the cloning efficiency. In the pre-test and the main experiment, the cells were exposed to A007-A017-A007 for 4 h with and without metabolic activation. Treatment was followed by an expression period of 7 days to fix the DNA damage into a stable *hprt* mutation. Toxicity was measured in the main experiments as relative cloning efficiency of the treated cultures relative to the relative cloning efficiency of the solvent control cultures.
Concurrent negative and solvent controls were employed. The known mutagens ethylmethane sulfonate (EMS) and 7,12-dimethylbenz(a)anthracene (DMBA) were used as positive controls without and with S9-mix, respectively.

Results
In most cases the recommended toxic range of approximately 10-20% survival compared to the concurrent negative controls was reached.
In both experiments a biologically relevant increase in mutant colony numbers was not observed up to the maximum concentrations tested which both without and with S9-mix. The mutant frequency generally remained well within the historical range of solvent controls, except at the maximum analyzable concentration of the first culture of the first experiment with metabolic activation. This isolated increase was not considered a relevant effect since it was neither reproduced in the parallel culture of the same experiment nor in the second experiment with metabolic activation, at comparable levels of cytotoxicity.
EMS (0.15 mg/ml) and DMBA (1.1 µg/ml) were used as positive controls and showed a distinct increase in induced mutant colonies.

Conclusion
Under the experimental conditions used, A007-A017-A007 did not induce mutations at the *hprt* locus of V79 cells and consequently is not genotoxic (mutagenic) in the gene mutation test.

Additional Ref.: N

In vitro micronucleus test in human lymphocytes

Guideline:	draft OECD 487	
Cells:	human peripheral blood lymphocytes from healthy, non-smoking 25-year old male volunteers	
Replicates:	duplicate cultures in a single experiment	
Test substance:	A007-A017-A007 (GTS25169)	
Solvent:	DMSO	
Batch:	GF814775	
Purity:	97.4% (HPLC, area at 254 nm)	
Concentrations:	4 h treatment	without S9-mix: 1.25, 2.5 and 5 µg/ml with S9-mix: 2.5, 5 and 10 µg/ml
	24 h treatment	without S9-mix: 1.25, 2.5 and 5 µg/ml
Treatment	48 h PHA followed by 4 and 20 h treatment with and without S9-mix 48 h PHA followed by 24 h treatment without S9-mix	

harvest time 24 h after the beginning of treatment
GLP: In compliance
Date: December 2008 - July 2009

The reaction product of p-phenylenediamine and 1-naphthol A007-A017-A007, dissolved in DMSO, was investigated in the absence and presence of metabolic activation for the induction of micronuclei in human erythrocytes. Blood from a healthy, 25 year old non-smoking male volunteer was used in this study. The mitogen phytohaemagglutinin (PHA) was included in the culture medium for 48 h in order to stimulate the lymphocytes to divide. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system.
A solubility test was conducted to determine the maximum soluble concentration or workable suspension using water. Since a workable stock concentration of 500 mg/ml was achieved in DMSO, DMSO was selected as the solvent. The dose selection for the micronucleus test was based on cell proliferation and cytotoxicity evaluated as an effect of A007-A017-A007 on the cytokinesis blocked proliferation index or replication index tested up to the maximum prescribed dose of 10 mM (= 3540 µg/ml).
The treatment period in the main test was either 4 h without and with S9-mix or 24 h continuously without S9-mix. Harvest time was 24 h after the beginning of treatment. Cytochalasin B (final concentration 6 µg/ml) was added during the recovery period in the cultures treated for 4 h and present continuously during treatment in the cultures treated for 24 h. Concurrent negative and solvent controls were employed. Mitomycine C, vinblastin and cyclophosphamide were used as positive controls.

Results
The doses for the micronucleus assay were chosen in a preliminary cytotoxicity assay. In this assay substantial toxicity (55±5% cytotoxicity relative to the solvent control) was observed at dose level 10.62 µg/ml in the non-activated 4 and 24 hour exposure groups and at dose level 35.4 µg/ml in the S9-activated 4 h exposure group.
The results for the positive and negative controls indicate that all criteria for a valid assay were met. Under the 3 treatment conditions both the results found for the cytokinesis blocked proliferation index as the % cytotoxicity indicated exposure of the human peripheral blood lymphocytes.
In all 3 treatment conditions, both in the absence and in the presence of S9-mix, biologically relevant, dose dependent and statistically significant increases in the percentage of human peripheral blood lymphocytes with micronuclei were not observed.

Conclusion
Under the experimental conditions used, A007-A017-A007 did not induce an increase in human peripheral blood lymphocytes with micronuclei *in vitro* both in the presence and absence of metabolic activation. Consequently A007-A017-A007 is not genotoxic (clastogenic and/or aneugenic) in human peripheral blood lymphocytes.

Additional Ref.: O

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

3.3.6.2.1 Genotoxicity tests with p-Phenylenediamine alone

Single cell gel electrophoresis assay (Comet assay)
(taken from Submission III, modified)

p-Phenylenediamine was tested in the single cell gel electrophoresis assay (Comet assay). A single oral (gavage) dose of 75 mg/kg bw (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 Comet assay; 50 nuclei per organ per mouse were examined.

Results
p-Phenylenediamine did not induce a biologically relevant induction of DNA damage in any of the tissues investigated at any time point.

Conclusion
Under the conditions of this test, p-phenylenediamine was not genotoxic in this single cell gel electrophoresis assay.

Ref.: 102

Comment
The data are from a publication in the open literature in which 30 compounds were tested in the Comet assay. The test was not conducted in compliance with GLP or OECD guidelines. The test has only limited value.

***In vivo* Micronucleus Assay**
(taken from SCCNFP/0129/99, adopted in 2002, modified)

Guideline:	/
Species/strain:	CD-1 mice
Group size:	5 male mice
Test substance:	p-phenylenediamine dihydrochloride
Batch:	/
Purity:	/
Vehicle:	Sterile distilled water
Dose level:	0, 25, 50 and 100 mg/kg bw
Route:	*ip* injection
Sacrifice times:	24, 48 and 72 h for all concentrations
GLP:	Not in compliance

p-Phenylenediamine has been investigated for the induction of micronuclei in bone marrow cells of mice. Test doses were based on the results of a preliminary study in which 3 out of 5 mice died after treatment with 200 mg/kg bw phenylenediamine. Therefore, in the main experiment 100 mg/kg bw was the highest dose tested. Positive and negative controls were included.

Results
The ratio PCE/NCE was slightly reduced at 24 h (100 mg/kg bw), at 48 h (25 mg/kg bw) and at 72 h (50 and 100 mg/kg bw). 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, was not observed at 72 h and, therefore, considered not biologically relevant.

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

Ref.: 113

Comment
The data are from a publication in the open literature. The test was not conducted in compliance with GLP or OECD guidelines. The test has only limited value and can only be used for confirmation purposes.

Genotoxicity tests taken from Submission III, modified

Bone marrow micronucleus test in rats

Guideline:	OECD 474
Species/strain:	Wistar rats
Group size:	5 rats/group/ sex
Test substance:	p-phenylenediamine
Batch:	1365
Purity:	99.8%
Dose level:	0, 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-[$U^{14}C$]-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 oxidizable 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 doses were based on a preliminary study on acute toxicity. As in a parallel GLP study oral treatment with 150 mg/kg bw 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 test 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 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 test no mortality occurred. Toxic signs following exposure were identical to that of the pre-experiment for toxicity but slightly decreased in severity 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 and the coloured urine indicated 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 at 24 h and 48 h after application was seen. This increase was within the range of the historical controls. Acridine orange staining demonstrated an increase of micronucleated bone marrow cells at 24 h but not at 48 h. The increases were again within the range of the historical controls.

Conclusion
Under the experimental conditions used p-phenylenediamine did not induce an increase in bone marrow cells with micronuclei and, consequently, is not genotoxic (clastogenic and/or aneugenic) in rats in this micronucleus test.

Ref.: 6 (suppl. data, 12/2005)

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:	0, 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 oxidizable 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 position 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 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 cells 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 (suppl. data, 12/2005)

3.3.6.2.2 Genotoxicity studies with Trimer A007-A017-A007 (reaction product of p-phenylenediamine and 1-naphthol)

***In vivo* unscheduled DNA synthesis (UDS) test**

Guideline:	OECD 486
Species/strain:	male Wistar HsdCpb:WU rats
Group size:	4 rats per dose
Test substance:	A007-A017-A007 (WR A021232)
Batch:	GF814775
Purity:	98% (HPLC, area at 254 nm)
Dose level:	0, 500 and 1000 mg/kg bw
Route:	oral gavage
Vehicle:	30% DMSO/70% PEG 400
Sacrifice times:	4 h and 16 h after dosing
GLP:	in compliance
Date:	February 2009 – January 2010

The test item A007-A017-A007 (reaction product of p-phenylenediamine and 1-naphthol) was investigated for the induction of unscheduled DNA synthesis (UDS) in hepatocytes of rats. A007-A017-A007 was formulated in 30% DMSO / 70% PEG 400. Test concentrations were based on the results of a pre-experiment on acute toxicity. Rats were treated orally up to 2000 mg/kg bw and examined for acute toxic symptoms at various intervals of 1, 2-4, 6, and 24 h after start of treatment. In the main experiment mice were exposed orally to 0, 500 and 1000 mg/kg bw. The volume administered orally was 10 ml/kg bw. The rats of all dose groups were examined for acute toxic symptoms at intervals of around 1, 2, 4 (4 h sacrifice only), and 16 h (16 h sacrifice only).
Hepatocytes for UDS analysis were collected approximately 4 h and 16 h after administration of A007-A017-A007. At least 90 minutes after plating the cells were incubated for 4 h with 5 µCi/ml ^{3}H-thymidine (specific activity 20 Ci/mmol) followed by overnight incubation with unlabelled thymidine. Evaluation of autoradiography was done after 14 days. UDS was reported as net nuclear grain: the nuclear grain count subtracted with the average number of grains in 3 nuclear sized areas adjacent to each nucleus. The percentage of cells in repair (defined as cells with a net grain count of at least +5) was calculated for each animal. Unscheduled synthesis was determined in 50 randomly selected hepatocytes on 2 replicate slides per rat from at least 3 treated rats.
Appropriate reference mutagens N,N´-dimethylhydrazine dihydrochloride (80 mg/kg bw) and 2-acetylaminofluorene (100 mg/kg bw) administered orally were used as positive controls, whilst negative control animals received the vehicle.

Results
The viability of the hepatocytes was not substantially affected by the treatment with A007-A017-A007 at any of the treatment periods or dose groups. The inter-individual variations obtained for the yield and the viability of the isolated hepatocytes were in the range of the historical laboratory control.

In the pre-experiment on acute toxicity with exposure up to 2000 mg/kg bw A007-A017-A007, reduction of spontaneous activity, ruffled fur and orange to dark red stained urine was seen at every dose tested up to 24 h after exposure. Additionally, at the highest doses tested 3 rats in the 1500 and 3 rats in the 2000 mg/kg bw dose group died. Occasionally apathy, abdominal position and eyelid closure was observed in these groups. Consequently, the maximum tolerated dose level of 1000 mg/kg bw was chosen to be suitable as top dose for the main experiment. In the main experiment reduction of spontaneous activity, ruffled fur and coloured urine was reported after A007-A017-A007 exposure. One rat died in the 1000 mg/kg bw dose group 16 h after treatment. The coloured urine of rats treated with A007-A017-A007 confirms its bioavailability and systemic distribution.
Neither a biological increase in mean net nuclear grain count nor in the percentage of cells in repair as compared to the untreated control was found in hepatocytes of any treated animal both for the 4 h and the 16 h treatment time.
The positive control substances (DMH, 80 mg/kg bw and 2-AAF, 100 mg/kg bw) revealed distinct increases in the number of nuclear and net grain counts, thus showing the validity of the test system used.

Conclusion
Under the experimental conditions reported A007-A017-A007 did not induce DNA damage leading to unscheduled DNA synthesis and, consequently, is not genotoxic in rats in the *in vivo* UDS test.

Additional Ref.: O

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 bw, 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

This study was carried out by the US National Toxicology Program.

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 bw by gavage
Route:	oral
Exposure:	10 days
GLP:	not in compliance

Pregnant NMRI mice (a group of 22 animals) were administered 30 mg/kg bw 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 bw) and vehicle was administered to controls (10 ml/kg bw). The F_1 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 at 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

This study was carried out by the US National Toxicology Program.

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 bw, Lab B: 6.4, 16 and 32 mg/kg bw
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 bw 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 bw 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: ≥ 99%
Dose: 30 mg/kg bw/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 bw/d PPD for 5 days. Positive control animals received 300 mg/kg bw/d urethane and vehicle control animals received 10 mg/kg bw/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 bw/day)	PPD (30 mg/kg bw/day)	Urethane (300 mg/kg bw/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 was 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.

The hair dye formulations were 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, 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: 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

Summary on carcinogenicity

The sensitivity of animal studies to detect carcinogenic substances is rather low. Thus, in experiments with groups containing 50 animals, the tumour incidence has to increase by more than 5% in order to be detected. As a consequence, the animals are generally exposed to much higher amounts of chemicals than 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 p-phenylenediamine together with hydrogen peroxide were identified. Three of the studies involved topical application to Swiss-Webster mice. All three 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 1B 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 p-phenylenediamine 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% p-phenylenediamine. 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. Two of the studies

were published by a group at the National Cancer Institute in Thailand in 1986. In both studies, there was a statistically significant increase in mammary gland tumours in female rats. The studies also included 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. 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 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 p-phenylenediamine + resorcinol + hydrogen peroxide. Furthermore, the concentrations of p-phenylenediamine were slightly higher in the studies from Thailand than in the study from USA.

IARC has classified p-phenylenediamine 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. p-Phenylenediamine 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. Such have also been tested in three experimental studies after topical application to rats. 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.

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 of mammary gland tumours was found both after topical application as well as after subcutaneous injection. In 1991, the SCC considered these studies as inadequate due to deficiencies in group size and duration. However, the SCCS is of the opinion that these results cannot be disregarded.

3.3.8. Reproductive toxicity

3.3.8.1. Reproduction toxicity

Taken from SCCP/0989/06

Hair dyes formulations containing 3% PPD mixed with an equivalent amount of hydrogen peroxide solution 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

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 bw/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 F_1 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 F_1 litter. No evidence of reduced fertility was recorded in the F_1 males. No compound-related changes were noted in the number of implantations, dead foetuses and resorptions.

Ref.: 24

A two-generation reproduction toxicity study has been performed in the 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 F0).
Treatment was continuous through growth, mating, gestation and lactation to the weaning of the F1A and F2B 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

In a recent study the testicular effect of 1% to 3% p-phenylenediamine (PPD) which should mimic the actual dosage available in most of the hair dyeing formulation was examined. In this sub-chronic study PPD was topically applied to male albino Sprague-Dawley rats at a dose of 0, 1, 2 and 3 mg/kg bw/day. The body weight was significantly decreased at 3 mg/kg bw/d. At 2 and 3 mg/kg bw/d a significant decrease in the absolute but not in the relative testes weight was observed. At these doses also a decrease in the total sperm count as well as a significant increase in the incidence of damaged seminiferous tubules was found (increase in germ cell apoptosis indicated by cellular morphology as well as loss of germinal layer and sloughing of testicular cellular layers). Elevation of lipid peroxidation product in the testicular tissue indicated potential oxidative stress that may be crucial in the induction of the apoptosis and further tissue injury in the PPD-treated rats.

Additional Ref.: Y

Comments of the SCCS
Some doubts are on the dosing since the authors equalize percentages and body weight related dosages. If this is true for example for a 130 g rat a 2 mg/kg bw/d dose would need a treatment with 0.26 mg, applied as 2% solution this would mean an application of 13 µl. However, the experimental details are not given. Also, no details of the changes of sperm morphology were published.
The further toxicity studies available did not support the findings of the study:

- A one generation study with Sprague-Dawley rats a 2.2% semi-permanent and a 2.2 % oxidative hair dye formulation (approximately 11 mg/kg bw/d) were topically applied for 10 weeks. No effects on P_0 body weights were found. No evidence for reduced fertility was found (see Ref. 24).

- In an oral subchronic toxicity study at 16 mg/kg bw/d PPD body weight was unaffected. Also weight and histopathology of the testes were unchanged (Ref. 121).

- In 90 day study in rabbits 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 bw of this mixture was applied for 1 hour without occlusion to three application sites on six animals of each sex. No dose-related changes were observed on weight gain, clinical chemistry, haematology, urinalysis or on examination of the tissues (including testes) at necropsy (Ref. 20).

Conclusion
The significance of the findings on testicular toxicity in this study remains unclear. Clarification might be achievable by a guideline generation study.

3.3.8.2. Teratogenicity

p-Phenylenediamine has been tested in one group of 25 Mice receiving a subcutaneous dose of 28 mg/kg bw 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 mixed with hydrogen peroxide before topical application at 2 ml/kg bw (corresponding to 20, 40, 60 and 80 mg/kg bw) 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

p-Phenylenediamine 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 bw/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 bw/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, p-phenylenediamine revealed no teratogenic or embryo-toxic effects.

Ref.: 93

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 slightly lower during the first 3 days of treatment at 10 and 20 mg/kg bw/day (not statistically significantly different), food consumption was not affected.
Foetal body weight was lower at 20 mg/kg bw/day, but the difference was statistically not significant. At this dose also retardation in ossification was seen (supraoccipital, sternebrae, thoracic vertebrae, metacarpals).

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

Ref.: 8 (suppl. data, 12/2005)

3.3.9. Toxicokinetics and metabolism

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 bw 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
Radiolabelled 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 bw) 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 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.

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

Human hepatic metabolism in vitro

Guideline:	/
Cells:	human hepatocytes, 4 male donors (cryo-preserved)
Test substance:	p-phenylenediamine dihydrochloride Sigma-Aldrich P6001
Radiolabelled 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 to N-acetylated derivatives by human hepatocytes. There was no evidence for the formation of mono-oxygenated metabolites in human hepatocytes, microsomes and by bacterially expressed human CYPs.

Ref.: 2, 3 (subm. 2005)

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_{0-24h} 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. 2005)

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 extracted 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. 2005)

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 [^{14}C-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 radioactivity 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 protein 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 radioactivity 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

In contrast to studies of human hepatic microsomal fractions (see Ref. 2), where no protein binding was found, covalent protein binding occurred in reconstructed human epidermis and in human hepatocytes.

New human exposure studies

Human systemic exposure to a 1.0% [^{14}C]-PPD-containing oxidative hair dye

In this study, a group of human subjects (N=16; 16 males; age range: 20 to 34 years) received a single application of an oxidative hair colouring product by professional hairdressers. The dye applied contained on-head concentrations of [^{14}C]-PPD (A007, 1.0%), resorcinol (A011, 0.5%) and m-aminophenol (A015, 0.5%). This study, along with the study below conducted with a 2% PPD-containing oxidative hair dye, permitted the investigation of the relationship between applied concentration and systemic exposure in humans. The exposure time was 30 minutes. Following hair colouring, the dye was rinsed off, and the hair was shampooed, dried and clipped. Skin tape stripping was performed on a representative area of exposed scalp surface. Urine was collected quantitatively for 48 hours post-exposure, blood samples were taken at pre-test, 2, 4, 6, 10, 24 and 48 hours. A protective cap was worn for 48 hours in order to collect residues in scalp skin scales. Under conditions of oxidative hair dye formation PPD (A007), resorcinol (A011) and m-aminophenol (A015) are known to form coloured dye trimers, such as A007-A015-A007 and A007-A011-A007, whereas PPD has been shown to be metabolised in human skin and excreted in human urine as N-mono acetyl-PPD and N,N'-di-acetyl-PPD (Ref. 13 subm V; Ref. 16, subm V; Ref. 17, subm V). Therefore, urine and plasma was analysed for PPD, N-monoacetyl-PPD, N,N'-diacetyl-PPD, the trimers A007-A015-A007 and A007-A011-A007, and the respective, potential N-mono- and N,N'-diacetylated metabolites of the dye trimers A007-A015-A007 and A007-A011-A007.

The overall mass balance obtained in this study was 96.21 ± 1.57%. The bulk of radioactivity was recovered in washing water and hair, which contained means of 64.6% and 30.2% of the applied radioactivity, respectively. Urinary excretion of [^{14}C]-PPD-equivalents represented 0.88 ± 0.46% of the applied radioactivity. In all plasma samples, PPD and N-monoacetyl-PPD levels were below their respective lower limit of quantification

(<500 pg/mL and <1000 pg/mL, respectively) whereas significant amounts of N,N'-diacetyl-PPD were found in the plasma from 2 to 48 hours. Plasma kinetic data yielded a C_{max} of 97.4 ± 61.5 ng/mL, a T_{max} of 2 hours, and a mean $AUC_{0-\infty}$ of 966 ± 575 ng * hr / mL, respectively.

Kinetic results of 1% PPD exposure are summarised in the following table.

Parameter	Units	Mean	SD	Min	Max
T_{max}	hr	2.0	0.0	2.0	2.0
C_{max}	ng eq/mL	97.4	61.5	20.9	234.0
$AUC_{(0-24h)}$	ng eq*hr/mL	762	401	410	1727
$AUC_{(0-\infty)}$	ng eq*hr/mL	966	575	228	1946
$T_{1/2}$	hr	7.8	5.1	3.5	25.8
Urinary excretion	% of dose	0.88	0.46	0.40	2.06

Some kinetic data indicate considerable differences between individuals (up to 11-fold).

Scalp S*tratum corneum* residues collected by skin stripping contained mainly PPD. Only N,N'-diacetylated PPD was found in the plasma; PPD or mono-acetylated PPD were not detected (0.5 and 1.0 ng/mL lower limits of quantification, respectively). Hair dye reaction products, i.e. the trimers A007-A015-A007 and A007-A011-A007 or their mono- or diacetylated metabolites were not detected in most plasma samples. A few samples occasionally contained traces of A007-A015-A007, mono- or diacetylated A007-A015-A007, slightly above their lower limits of quantification (LLOQs ranging from 0.1 – 0.32 ng/mL), suggesting negligible systemic exposure to these compounds.

Urine samples mainly contained N,N'-diacetylated PPD (>99% of the substances found); some samples also contained very low levels of PPD (mean <0.3%) or mono-acetylated PPD (mean <0.2%). Hair dye reaction products A007-A015-A007 and A007-A011-A007 were generally not detectable in urine samples. In a few samples, traces of A007-A015-A007, A007-A011-A007 and mono-acetylated A007-A015-A007 were detected in the 0-12 hour urine samples. When comparing human systemic exposure levels of reaction products with that of N,N'-diacetyl-PPD, the principal metabolite of PPD, exposure to reaction products was 3 to 4 orders of magnitude lower.

Kinetic results of N,N'-diacetyl-PPD after exposure to 1% PPD are summarised in the following table:

Parameter	Units	Mean	SD	Min	Max
T_{max}	hr	2.5	1.2	2.0	6.0
C_{max}	ng/mL	118.2	92.0	24.2	388.1
$AUC_{(0-48h)}$	ng*hr/mL	939.7	697.5	227.2	2848.5
$AUC_{(0-\infty)}$	ng*hr/mL	962.7	690.3	250.2	2858.2
$T_{1/2}$	hr	6.09	1.13	3.98	7.92
Urinary excretion$_{(0-48\ h)}$	µg	3067	2231	835	9352

Some kinetic data indicate considerable differences between individuals (up to 16-fold).

Ref.: 14, subm V

Comment
N,N'-diacetyl-PPD as the almost exclusive metabolite of PPD in plasma and urine suggest a predominant role of N-acetylation in the inactivation of PPD in human skin. Furthermore, the considerable differences of kinetic data and urinary excretion of N,N'-diacetyl-PPD

between individuals need attention (see summary of studies on metabolism and toxicokinetics below).

Human systemic exposure to a 2.0% [^{14}C]-PPD-containing oxidative hair dye

In this study, a group of human subjects (N=16; 12 males, 4 females; age range: 20 to 32 years) received a single application of an oxidative hair dye by professional hairdressers. The dye applied contained on-head concentrations of [^{14}C]-PPD (A007, 2.0%), i.e. the maximal PPD concentration permitted in the EU, as well as resorcinol (A011, 1.0%) and m-aminophenol (A015, 1.0%). The exposure was for 30 minutes. Following hair colouring, the dye was rinsed off, and the hair was shampooed, dried and clipped. Urine was collected quantitatively for 48 hours, and blood samples were taken at pre-test, 2, 4, 6, 10, 24 and 48 hours. A protective cap was worn for 48 hours in order to collect residues in skin scales.

The overall mass balance obtained in this study was 94.30 ± 3.01%. The bulk of radioactivity was recovered in washing water and coloured hair, which contained a mean of 61.5% and 31.7% of the applied radioactivity, respectively.

Urinary excretion represented 0.72 ± 0.25% of the applied radioactivity. Plasma kinetic data yielded a C_{max} of 132.6 ± 52 ng/mL, a T_{max} of 2 hours, and a mean $AUC_{0-\infty}$ of 1415 ± 592 ng * hr/mL, respectively.

Kinetic results of 2% PPD exposure are summarised in the following table.

Parameter	Units	Mean	SD	Min	Max
T_{max}	hr	2.0	0.0	2.0	2.0
C_{max}	ng eq/mL	132.6	52	46.0	219,5
$AUC_{(0-24h)}$	ng eq*hr/mL	1169	485	441	2182
$AUC_{(0-\infty)}$	ng eq*hr/mL	1415	592	554	2716
$T_{1/2}$	hr	7.8	2.1	4.5	12.5
Urinary excretion	%	0.72	0.25	0.31	1.43

Some kinetic data indicate marked differences between individuals (up to 6-fold).

Ref.: 15, subm V

Summary of studies on metabolism and toxicokinetics

Human hepatocytes *in vitro* were found to form the 2 acetylated PPD derivatives. In microsomes, no experimental evidence was seen with regard to the formation of monoxygenated products of PPD. Following incubation of PPD with recombinant human CYPs derived from a bacterial expression system also 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.

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 bw, 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 2.0% [^{14}C]-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 di-acetylated metabolite of PPD. No difference of the metabolic profile was found in the NAT2 genotype subgroups (low and intermediate acetylators).

Two new studies in human subjects were performed that measured the magnitude of human systemic exposure to [^{14}C]-PPD_{eq}. These studies were conducted in order to give insights into systemic exposure associated with the use of PPD-containing hair colouring products under realistic conditions. In one of these studies, in addition to measuring [^{14}C]-PPD_{eq} levels, the systemic exposure to specific substances (parent compound/metabolites) was identified after an application of a commercial oxidative PPD-containing hair colouring product. The analytical methods used (LC-MS/MS) permitted the identification and quantification of human systemic exposure to these compounds at low levels. The data suggest a predominant role of N-acetylation in human skin. Furthermore, the differences of kinetic data and urinary excretion of N,N'-diacetyl-PPD between individuals are higher compared with similar studies (e.g., toluene-2,5-diamine (A005, SCCS/1479/12)). Apart from the different absorption of PPD in the skin of human individuals, the data suggest an influence of NAT1 polymorphisms in human skin. In contrast, no influence of NAT2 genotype subgroups on the metabolic profile was found. This is consistent with a minor role of NAT2 after dermal exposure to PPD, as NAT2 is relevant in human liver and gut, but not in human skin, the organ primarily exposed in these studies. Overall, these results indicate that the relevant human systemic exposure agent after hair dyeing with a [^{14}C]-PPD-containing oxidative hair dye is N,N'-diacetyl-PPD, which is considered a non-toxic metabolite as it is apparently not hydroxylated and has no mutagenic potential in *in vitro* tests. For the purpose of a toxicokinetic-based margin of safety calculation discussed below, plasma AUC for PPD_{eq} (primarily to the N,N'-diacetylated metabolite) in humans after hair dye use can be compared to plasma AUC in rats at a PPD dose corresponding to the NOAEL.

3.3.10. Photo-induced toxicity

3.3.10.1. Phototoxicity / photoirritation and photosensitisation

No data submitted

3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity

No data submitted

3.3.11. Human data

No data submitted

3.3.12. Special investigations

No data submitted

3.3.13. Safety evaluation (including calculation of the MoS)

- **Evaluation based on Margin of Safety (MoS)**

In 2006 the SCCP concluded that, based on the classical MoS approach, the safety of p-phenylenediamine used as an oxidative hair dye at an applied concentration of 2% could not be warranted. For the calculation of the MoS, a NOAEL of 4 mg/kg bw/d was used. This NOAEL was derived from a 90 days oral toxicity study in which mean absolute and body-weight related liver and kidney weights were significantly increased for males and females, at 8 mg/kg bw/d and 16 mg/kg bw/d respectively, and minimal myodegeneration on skeletal muscle were observed in one male and one female of the high dose group. The MoS resulted in 77. The applicant proposed to base the safety on the comparison of AUCs (area under curve) described below. In the recent submission of 2011, the applicant, based on the expert review of the 90 days toxicity study, proposed that 4 mg/kg bw/d should be considered as a NOEL and not as a NOAEL, as the increase in body-weight related liver and kidney weights observed were considered mild modifications, not associated with histopathological or clinical pathology changes and adaptive, thus non–adverse. Therefore, the NOAEL from this study should then be 16 mg/kg bw/d (the highest dose tested).

The SCCS agrees to this judgement of kidney and liver effects observed and that in the 90 days toxicity study, 4 mg/kg bw/d could be considered as a NOEL instead of a NOAEL for these effects. The increase in body-weight related liver and kidney weights at the dose of 8 and 16 mg/kg bw/d, even if statistically significant remains still slight. Moreover they were not associated with histopathological changes or clinical chemistry alterations. However, the observed myodegenerative effects also need to be considered. It is well known that PPD and some substituted derivatives can induce myotoxicity (e.g. even after 3 days of administration of 36.2 mg/kg bw, see Ref. I). This myodegeneration in the skeletal muscle was also noted in the 28-day study in 3 females given 40 mg/kg bw/d (Ref. 120). Minimal myodegeneration on skeletal muscle was noted in the 90-day study on 1 male and 1 female of the high dose group (16 mg/kg bw/d). Although this was also observed in the respective control group a relation to PPD treatment cannot be excluded. Therefore, the SCCS sets the NOAEL for subchronic toxicity at 8 mg/kg bw/d. For the MoS calculation a skin surface area of 580 cm^2 was used (see Notes of Guidance).

CALCULATION OF THE MARGIN OF SAFETY

p-phenylenediamine

Absorption through the skin	**A (mean + 1SD)**	**=**	**4.47 µg/cm²**
Skin Area surface	**SAS**	**=**	**580 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**2.6 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.04 mg/kg bw/d**
No Observed Adverse Effect Level (subchronic toxicity, oral, rat)	**NOAEL**	**=**	**8 mg/kg bw/d**
MOS		**=**	**200**

- **Evaluation based on toxicokinetics**

In its 2006 opinion, the SCCP also calculated a toxicokinetic data based MoS by comparing plasma AUC in rat following oral administration of PPD at the NOAEL (4 mg/kg bw/d) to plasma AUC in human following dermal application to hair dye containing PPD.

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 bw) 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_{0-24h} 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_{0-24h} 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 bw/day) yields a toxicokinetic-based safety margin of **16.3-fold.**

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). When AUC values are available from relevant human and animal kinetic studies, these values can be directly used for the calculation of the MoS. When these AUCs are compared, a MoS of 25 between the AUC of rat and human is considered sufficient since in this case the interspecies factor for toxicokinetics can be set at 1.
The MoS of 16.3 was therefore considered too low by the SCCP to support the safety of this compound.

Additional Ref.: D, E

Present evaluation

For the present submission, two new exposure studies were performed in humans to measure systemic exposure to PPD and its metabolite following real conditions of exposure in hair-dyeing.

In addition, a number of studies have investigated the plasma toxicokinetic profile in rats given oral doses of radiolabelled PPD. In the first study (Ref. 2, subm V) male and female adult Sprague-Dawley rats were given a single oral dose of 4 mg/kg bw [^{14}C]-PPD by gavage. The area under the plasma concentration-time curve (AUC) between 0 h and 24 h was estimated by the trapezoidal rule and found to be 10842 (ng-eq/g)*h in males and 10797 (ngeq/g)*h in females. Both the C_{max} and AUC are probably slight underestimates because the true C_{max} probably occurred prior to the first sample and also the AUC was not extrapolated to infinity. Extrapolation from the concentration at 24 h to infinity, using the elimination rate between 12 h and 24 h (-0.086 h^{-1} in males and -0.065 h^{-1} in females) would add 181 (ng-eq/g)*h in males and 332 (ng-eq/g)*h in females; thus the AUCs extrapolated to infinity are 11023 (ng-eq/g)*h in males and 11129 (ng-eq/g)*h in females. Overall, expressed per mg/kg bw of PPD administered, the C_{max} and AUC data are equivalent to approximately 1000 ng-eq/g and 2,750 (ng-eq/g)*h, respectively.

In the second study (Ref. 3, subm V) male and female adult Sprague-Dawley rats were given a single oral dose of 6.45 mg/kg bw [^{14}C]-PPD by gavage. In this experiment blood samples were taken from 3 rats of each sex at 0.5, 2, 4 and 8h after the single gavage dose and a different 3 rats of each sex at 1, 12 and 24 h after the single gavage dose.

Comparison of these data with those following a dose of 4 mg/kg bw it appears that there may have been slightly slower elimination. The area under the plasma concentration-time curve (AUC) between 0 h and 24 h was estimated by the trapezoidal rule and found to be 24847 (ng-eq/g)*h in males and 27304 (ngeq/g)*h in females. Extrapolation from the concentration at 24 h to infinity, using the elimination rate between 12 h and 24 h (-0.110

h^{-1} in males and -0.091 h^{-1} in females) would add 373 (ng-eq/g)*h in males and 750 (ng-eq/g)*h in females; thus the AUCs extrapolated to infinity are 25220 (ng-eq/g)*h in males and 28054 (ng-eq/g)*h in females (average 26637 (ng-eq/g)*h). Overall, expressed per mg/kg bw administered, the C_{max} and AUC data are equivalent to approximately 1100 ng-eq/g and 4200 (ng-eq/g)*h, respectively.

The two studies show good consistency in the rate and extent of absorption, the routes of elimination and the plasma concentration time curves. There is no indication of a sex difference in the disposition of PPD in rats at either dose. The slightly higher dose-adjusted AUC at 6.45 mg/kg bw compared with 4 mg/kg bw indicates the possibility of slower elimination with increase in dose.

Calculation of the MOE for other doses and studies in rats (see Table below) is based on dose adjustment of the AUC in rats, assuming a linear relationship in rats between external dose and systemic exposure. However, the dose-adjusted $AUC_{0\text{-infinity}}$ of radioactivity is higher at 6.45 mg/kg bw than at 4 mg/kg bw indicating the possibility of dose-dependent kinetics in rats. Therefore in the following analyses AUC for higher doses has been calculated based on both dose-adjusted values for AUC0-infinity in rats. At the dose of 4 mg/kg bw AUCs of 11023 (ng-eq/g)*h in males and 11129 (ng-eq/g)*h in females giving an average of 11076 (ng-eq/g)*h and a dose-adjusted AUC of 2769 (ng-eq/g)*h per mg/kg bw were reported. At the dose of 6.45 mg/kg bw AUCs of 25220 (ng-eq/g)*h in males and 28054 (ng-eq/g)*h in females giving an average of 26637 (ng-eq/g)*h and a dose adjusted AUC of 4130 (ng-eq/g)*h per mg/kg bw were reported.

The systemic human exposure used in calculation of the MOE is based on the $AUC_{0\text{-infinity}}$ for total radioactivity expressed as PPD equivalents (ng-eq/g*h) using the data from the recent study of Meuling and de Bie (Ref. 14, subm. V) in which 1% PPD was applied on head.

Margins of exposure (MOE) derived based on the systemic exposure of rats treated with PPD in toxicity studies and humans exposed to hair dyes providing either 2% or 1% PPD on head.

Dose in rats (mg/kg bw)	**$AUC_{0\text{-infinity}}$ in rats** (based on data from 4 mg/kg bw kinetic study)	**$AUC_{0\text{-infinity}}$ in humans** (2% on head)	**MOE**	**$AUC_{0\text{-infinity}}$ in humans** (1% on head)	**MOE**
Gavage dose of 4 (NOAEL used by the SCCP)	11076	1415	7.8	966	11.5
Gavage NOAEL of 8	22152	1415	15.6	966	22.2
Gavage NOAEL of 16	44304	1415	31.3	966	45.9
Dietary NOEL of 50	138450	1415	97.8	966	143.3
	$AUC_{0\text{-infinity}}$ in rats (based on data from 6.45 mg/kg bw kinetic study)	**$AUC_{0\text{-infinity}}$ in humans** (2% on head)	**MOE**	**$AUC_{0\text{-infinity}}$ in humans** (1% on head)	**MOE**
Gavage dose of 4 (NOAEL used by the SCCP)	*16519*	*1415*	***11.6***	*966*	**17.1**
Gavage NOAEL of 8	***33038***	***1415***	***23.3***	966	34.2
Gavage NOAEL of 16	66076	1415	46.7	966	68.4
Dietary NOEL of 50	206488	1415	145.9	966	213.8

Comment of the SCCS

For the toxicokinetics-based risk assessment, the SCCS considered that the plasma AUC in humans from the new studies should be compared to the plasma AUC in rats corresponding to the NOAEL of 8 mg/kg bw/d in the subchronic toxicity study.

For the assessment of a toxicokinetic based MOS, the following data were used:

- NOAEL 8 mg/kg bw/d
- The $AUC_{0\text{-infinity}}$ value in rats of 33038 PPD equivalents (ng-eq/g)*h based on data from 6.45 mg/kg bw kinetic study)
- The $AUC_{0\text{-infinity}}$ value in humans of 1415 PPD equivalents (ng-eq/g)*h using the data for 2% PPD applied on head

From these data a toxicokinetic based MOS of 23.3 is derived. As described above, the SCCS considers that for the toxicokinetics-based risk assessment approach, a MoS of 25 should be achieved. The toxicokinetic based MoS of 23.3 is borderline. However, in view of the intermittent exposure to PPD in oxidative hair dyes and the fact that human systemic exposure through hair dyeing is mainly to the de-toxified metabolite of PPD, N,N'-diacetyl PPD, no concern regarding systemic toxicity is raised.

3.3.14. Discussion

Physico-chemical specifications
No data on stability in test solutions and in typical hair dye formulations were 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 bw died within 3 h. The animal treated with 50 mg/kg bw showed clinical signs (lacrimation, swelling of conjunctivae, gait, tremor, subdued behaviour and/or piloerection). 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 the skeletal muscle, with a rhabdomyolysis being experimentally observed following oral application in the rat at levels down to 16 mg/kg bw. Myocardial damage is also described with PPD-related hair dye poisonings in humans.

- From a 90 day study, a NOAEL of 4 mg/kg bw/d was obtained and was used as the basis for the safety evaluation by the SCCP in 2006. In an expert review submitted in the recent dossier it was argued that liver and kidney weight increases at 8 and 16 mg/kg bw/d are the only changes in this study which are considered to be related to treatment with PPD. Such organ weight changes without associated clinical chemistry alterations and without any corresponding histopathological changes were considered to be adaptive in nature and non-adverse. The applicant concluded for this study that the no observed effect level (NOEL) is 4 mg/kg bw/d and the no observed adverse effect level (NOAEL) is 16 mg/kg bw/d.

The SCCS agrees to this judgement of kidney and liver effects observed. However, myodegenerative effects also need to be considered. It is well known that PPD and some substituted derivatives can induce myotoxicity (e.g. even after 3 days of administration of 36.2 mg/kg bw, see Ref. I). This myodegeneration in the skeletal muscle was also noted in the 28-day study in 3 females given 40 mg/kg bw/d (Ref. 120). Minimal myodegeneration

on skeletal muscle was noted in the 90-day study on 1 male and 1 female of the high dose group (16 mg/kg bw/d). Although this was also observed in the respective control group a relation to PPD treatment cannot be excluded. Therefore the SCCS sets the NOAEL for subchronic toxicity at 8 mg/kg bw/d.

In a gavage developmental toxicity study in rats the NOEL of maternal toxicity is 5 mg/kg bw/day whereas the NOAEL of developmental toxicity is 10 mg/kg bw/day. Studies on reproductive toxicity were only performed with PPD in hair dye formulations. The results were not consistent.

Irritation, sensitisation
PPD was not irritant or corrosive for the skin and the eye when applied in a 2.5% aqueous solution.

PPD was shown to be an extremely potent contact allergen in animals.

p-Phenylenediamine is a frequent allergen in humans, which is of concern. It is recognized that allergic reactions to it may be severe; it is an extreme sensitiser. Unlike other sensitising hair dye chemicals, p-phenylenediamine has/is used during routine diagnostic patch testing in clinical practice. Therefore, the relevance and importance of PPD as a sensitiser for the consumer is well documented.

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 bw, 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*h/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 were found to form the 2 acetylated PPD derivatives. No experimental clues were seen with regard to the formation of mono-oxygenated products in microsomes as well as in recombinant human CYPs from bacteria. In hepatic 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. detoxified metabolites which were also found to be non-mutagenic (see below).

Mutagenicity
In COLIPA Submission III (2000) and a previous opinion of the SCCNFP (2002), the results of a number of genotoxicity studies were reviewed. 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 and exposure times in the respective test systems exceeded by far the time of human contact with p-phenylenediamine-containing oxidative hair dyes.

Moreover, these tests were not conducted in compliance with GLP and predominantly not performed according to the standard procedures of the OECD guidelines. Obviously, these tests have only limited value and can only be used for supporting evidence. For the safety dossier on p-phenylenediamine, a new battery of genotoxicity studies under stringent conditions concerning test material and test protocols was generated. In this new battery, consisting of studies performed after 2005, p-phenylenediamine has been tested sufficiently for the 3 genetic endpoints (gene mutations, structural and numerical chromosomal aberrations). The final conclusion of this discussion is based on these "new" tests.

Testing under *in vitro* conditions p-phenylenediamine alone resulted in different results. In an *in vitro* gene mutation test in bacteria a positive result in strain TA98 after p-phenylenediamine treatment both with and without S9-mix was found. p-Phenylenediamine exposure did not result in an increase of the mutant frequency in an *in vitro* gene mutation assay in mammalian cells at the *hprt* locus. A gene mutation test with mouse lymphoma cells (*tk* locus), reported in the SCCNFP opinion (2002), was positive. p-Phenylenediamine was positive in an *in vitro* micronucleus test.
The newly submitted *in vivo* unscheduled DNA synthesis test was negative and thus did not confirm the positive results from the *in vitro* gene mutation tests. Also in an *in vivo* COMET assay (not in compliance with GLP) in mice, several tissues including bladder epithelium were analysed. No DNA damage was detected. An *in vivo* micronucleus test (not according to OECD guideline and GLP) in mice (*ip* administration) was negative. However, in a newly submitted *in vivo* micronucleus test in rats, the increases found in the number of cells with micronuclei were all within the range of the historical controls and, consequently, did not confirm the positive results found in the *in vitro* micronucleus test. The results from these studies indicate that p-phenylenediamine alone has no genotoxic potential

In old studies reported in the SCCNFP opinion (2002), the reaction product of p-phenylenediamine and resorcinol gave contradictory results in gene mutation tests with bacteria; in one test the reaction product induced an increase in the number of revertants whereas in a second test it was negative. A mixture of p-phenylenediamine with hydrogen peroxide induced gene mutations in a gene mutation test in bacteria but not in mammalian cells and an increase in cells with chromosome aberrations in a chromosome aberration test. In contrast, although not tested according to current standards, a mixture of p-phenylenediamine, hydrogen peroxide and the coupler resorcinol did not result in gene mutations in bacteria or mammalian cells nor in an increase in cells with chromosome aberrations. *In vivo* studies with these reaction products or mixtures have not been submitted. The results from these old studies may indicate that p-phenylenediamine once together with a coupler has no genotoxic potential.

The reaction product of p-phenylenediamine with 1-naphthol was also tested for its genotoxic potential. This reaction product was positive in a gene mutation test in bacteria inducing mutations in *Salmonella typhimurium* strains TA98 and TA1537. In a gene mutation test in mammalian cells (*hprt* locus) treatment with the reaction product did not result in an

increased mutant frequency. Exposure to this reaction product did not induce an increase in human peripheral blood lymphocytes with micronuclei *in vitro.* The unscheduled DNA synthesis test performed in order to explore the *in vivo* genotoxicity potential of the reaction product in light of the results obtained in *in vitro* gene mutation assays - was negative. Consequently, the reaction product can be considered to have no *in vivo* genotoxic potential.

In recent studies, 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.

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

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 of mammary gland tumours was found both after topical application as well as after subcutaneous injection. In 1991, the SCC considered these studies as inadequate due to deficiencies in group size and duration. However, the SCCS is of the opinion that these results cannot be disregarded.

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 and rats. The sensitivity of these studies may have been low as they did not respond to hair dye formulations containing known carcinogens.

On the basis of the present data on carcinogenicity, no conclusions with regard to carcinogenicity of PPD in oxidative hair dye formulations can be drawn.
However, on the basis of the toxicokinetic and mutagenicity data, it is unlikely that PPD in oxidative hair dye formulations would pose a carcinogenic risk for the consumer.

Safety assessment
A Conventional approach based on NOAEL: In a subchronic toxicity study the NOAEL was 8 mg/kg bw/d 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 μg/cm². This leads to a margin of safety of 200. Even if the NOEL for maternal toxicity of 5 mg/kg bw/d would have been used, the resulting MoS would be sufficient (125).

B Toxicokinetic based approach: In human volunteers (N=16), hair dyeing with a 2.0% [^{14}C]-PPD-containing hair dye produced an $AUC_{0\text{-infinity}}$ value of 1415 PPD equivalents (ng-eq/g)*h. This human plasma AUC was compared with that of rats at the NOAEL of a subchronic oral toxicity study (8.0 mg/kg bw/day) based on data from the 6.45 mg/kg bw kinetic study. The $AUC_{0\text{-infinity}}$ value in rats was 33038 PPD equivalents (ng-eq/g)*h. From these data a toxicokinetic-based safety margin of 23 was calculated.

The toxicokinetic based MoS is borderline. However, in view of the intermittent exposure and the fact that human exposure through hair dyeing is mainly to N,N'-diacetyl PPD mainly formed in human skin, no concern regarding systemic toxicity is raised.

4. CONCLUSION

For the final safety assessment of p-phenylenediamine several aspects were taken into account:

- The generally accepted approach for systemic toxicity (MOS approach) according to the Notes of Guidance results in a MoS of 200. 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 extrapolated AUC in rats resembling a peroral dosage of 8 mg/kg bw/day (corresponding to the NOAEL) was compared to the AUC in humans following application of a hair dye containing ^{14}C-labelled PPD. In this case, a safety margin of 23 was obtained which is considered borderline. However, in view of the intermittent exposure and the fact that human exposure through hair dyeing is mainly to N,N'-diacetyl PPD (a non-mutagenic detoxified metabolite), no concern regarding systemic toxicity is raised, when PPD is used in oxidative hair colouring products at on-head concentrations of up to 2.0%.

- On the basis of the available data from carcinogenicity studies alone, no conclusion with regard to carcinogenicity of p-phenylenediamine in oxidative hair dye formulations can be drawn. However, on the basis of the toxicokinetic and mutagenicity data, it is unlikely that p-phenylenediamine in oxidative hair dye formulations would pose a carcinogenic risk for the consumer.

- p-Phenylenediamine was shown to be an extremely potent contact allergen in animals. p-Phenylenediamine is also an important and frequent allergen in consumers. It is recognized that allergic reactions to it may be severe. p-Phenylenediamine is an extreme sensitiser. Unlike other sensitising hair dye chemicals, p-phenylenediamine has been/is used during routine diagnostic patch testing in clinical practice, and therefore, the importance of this molecule as a sensitiser for the consumer is very well documented. The continued use of p-phenylenediamine in hair dyes remains a considerable concern for consumer safety.

Additionally, exposure to PPD from so-called temporary tattoos may also result in sensitisation in consumers.

5. MINORITY OPINION

Not applicable

6. REFERENCES

Submissions I (1979), II (1987), III (2000)

Toxicology summaries

American Conference of Governmental Industrial Hygienists (1989) p-Phenylenediamine ACGIH Monograph, 474, 8-16

References from previous submissions (Submissions I & II); epidemiology section and discussion from submission III

1. Ames, B. et al (1975) Hair Dyes are Mutagenic: Identification of a Variety of Mutagenic Ingredients Proc. Nat Acad. Sci. 72,2423

2. An-Ex, Cardiff, UK (1997). Skin penetration of p-Phenylenediamine from hair dye formulations - In vitro assessment. Report n° CO5/19X/97
3. Antti Seppala. Evaluation of toxicity and carcinogenicity of 12 hair dye formulations. Eppley Institute for Research in Cancer, 1978
4. Angelini G. et al (1985) Contact Dermatitis Due to Cosmetics. J. Appl. Cosmetol. 3, 223-236
5. Antti Seppala (1978) Evaluation of Toxicity and Carcinogenicity of 12 Hair Dye Formulations. Eppley Institute for Research in Cancer
6. Antunes M.A. et al (July 1998) Acute Dermatitis after Application of Hair Dyes. Fourth Congress of the European Society of Contact Dermatitis, Helsinki, P-9
7. Annstrong, D.K.B. et al (1999) Occupational sensitisation to p-Phenylenediamine: a 17-year review. Contact Dermatitis 41, 348-349
8. Ashraf W. et al (1994) Systemic Paraphenylenediamine (PPD) Poisoning: A Case Report and Review. Human & Experimental Toxicology 13, 167-170
9. Averbukh, Z. et al (1989) Rhabdomyolysis and Acute Renal Failure Induced by Paraphenylenediamine. Human Toxicol. 8, 345-348
10. Basketter D.A. and Goodwin, B.F.J. (1988) Investigation of the prohapten concept. Contact Dermatitis, 19, 248-53
11. Basketter, D. et al (1994). The performance of the local lymph node assay with chemicals identified as contact allergens in the human maximization test
12. Baud, F. et al (1983) Rhabdomyolysis in Para-phenylenediamine Intoxication. Lancet 2 (8348), 514
13. Biodynamics Inc. (1977) A modified Segment II Teratology Study of Hair Dyes in Mice
14. Blijleven (1977) Mutagenicity of Four Hair Dyes in Drosophila melanogaster. Mutation Research 48, 181-6
15. Blijleven (1981) Mutation Research 90, 137-41
16. Bracher, M. et al (1990) Studies on the Potential Mutagenicity of p-phenylenediamine in oxidative hair dye mixtures. Mutation Research 241, 313-23
17. Brown, J. et al (1987) Chronic Renal Failure Associated with Topical Application of Paraphenylenediamine. British Medical Journal 294. 155
18. Buehler, E.V. (1985). A rationale for the selection of occlusion to induce and elicit delayed contact hypersensitivity in the Guinea Pig. Curr. Probl. Derm. 14, 39-58
19. Burnett, C. et al. (1975) Long-term Toxicity Studies on Oxidation Hair Dyes. Fd. Cosmet. Toxicol. 13, 353
20. Burnett, C. et al. (1976) Teratology and Percutaneous Toxicity Studies on Hair Dyes. Journal of Toxicology and Environmental Health 1, 1027-1040
21. Burnett, C. et al. (1977) Dominant Lethal Mutagenicity Studies on Hair Dyes. Journal of Toxicology and Environmental Health 2, 657-662
22. Burnett C. Environmental Mutagen Society meeting Colarado Springs (1977)
23. Burnett, C. et al. (1979) Mutagenicity studies on urine concentrates from female users of dark hair color product. Drug Chem. Toxicol. 2(3), 283-93
24. Burnett C et al. (1981). Heritable translocation study on two hair dye formulations. Fundam Appl Toxicol 1, 325-328
25. Burnett, C. et al. (1982) The Effect of Dimethylsulfoxide on the Mutagenicity of the Hair Dye p-Phenylenediamine. Mutation Research 103, 1-4
26. Burnett C M et al. (1988). Multigeneration reproduction and carcinogenicity studies in Sprague-Dawley Rats exposed to oxidative hair-colouring formulations containing p-Phenylenediamine and other aromatic amines. Fd Chem Toxic 26(5), 467-474
27. Caspary W. J., Langenbach R., Penman B. W., Crespi C., Myhr B. C., Mitchell A. D. Mut. Res. 196: 61-81 (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
29. CIR Expert Panel (1985) Final Report of the safety assessment of p-Phenylenediamine. Journal of the American College of Toxicology, 4(3), 203-266
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).

31. Dethloff, L A. et al. (1996) Toxicological Comparison of a Muscarinic Agonist Given to Rats by Gavage or in the Diet. Food Chem. Toxicol. 34 (4), 407-22
32. Dooms-Goossens, A. et al. (1989) Comparative Patch Testing with PPD-Base and PPD-Dihydrochloride: Human and Animal Data Compiled by the European Environmental Contact Dermatitis Research Group in : Current Topics in Contact Dermatitis, Springer-Vedag, , part 3, 281-285
33. Dunkel V. C., Schechtman L. M., Tu A. S., Swak A., Lubet R. A., Cameron T. P. Environ. Mol. Mut. 12: 21-31 (1988).
34. Dybing E., Thorgeirsson S. S. Biochem. Pharmacol. 26: 729-34 (1977)
35. Edman, B. et al. (1982) Trends and forecasts for standard allergens in a 12-year patch test material. Contact Dermatitis 8, 95-104
36. El-Ansary, E. H. et al. (1983) Systemic Toxicity of Para-phenylenediamine. Lancet 1(8337), 1341
37. Elsner, P. (Feb. 1995) Trends in Contact Sensitization in Zurich, Switzerland. American Contact Dermatitis Society Annual Meeting, Abstract 8, New Orleans, Louisiana
38. Fregert, S. et al. (1968) Epidemiology of contact dermatitis Trans St. John's Hospital Dermatol. Soc. 55, 17-35
39. Gad, S. et al (1986). Development and validation of an alternative dermal sensitization test: the mouse ear swelling test (MEST). Toxicol Appl Pharmacol 84, 93-114
40. Garner R. C., Nutman C. A. Mut. Res. 44: 9-19 (1977)
41. Gentile J. M., Gentile G. J., Plewa M. J. Mut. Res. 188: 185-96 (1987)
42. Giles, A. L et al. (1976) 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
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
44. Goh, C. L. (1987) Allergic Contact Dermatitis from Cosmetics. J. of Dermatol 14, 248-252
45. Gola, M. et al. (1992) GIRDCA Data Bank for Occupational and Environmental Contact Dermatitis (1984 to 1988) American Journal of Contact Dermatitis 3, 179-188
46. Hammershoy, O. (1980) Standard patch test results In 3,225 consecutive Danish patients from 1973 to 1977. Contact Dermatitis 6, 263-268
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.
48. Haskell Laboratory for Toxicology and Industrial Medicine, Newark Delaware USA (1992). Subchronic oral neurotoxicity study of H.18508 in Rats. Report 854-91
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 pfenyllendiamin 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 pfenylendiamin 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. Imalda, K. et al. (1983). Carcinogenicity and toxicity tests on p-Phenylenediamine in F344 Rats. Toxicol. Letter 16, 259-269
54. International Research and Development Corporation (1979) Lifetime Chronic Toxicity/Carcinogenesis Study in Rats. Final report
55. Ioannou, Y.M. et al. (1985). p-Phenylenediamine dihydrochloride: comparative disposition in male and female rats and mice. J of Toxicol and Environ Health 16, 299-313

56. Ishihara, M. et al. (1983) Basic Studies on Contact Dermatitis Due to Hair Colorings and Cold Permanent Wave Solutions JNCI 70,443-446
57. Jouhar, A. J. (1979) Bristol Myers Company Limited
58. Kalopissis, G. (1986) Toxicology and Hair Dyes in: The Science of Hair Care, Zviak, C (ed.), Marcel Dekker, New York, 287-308
59. Kerckaert, G.A., Leboeuf R.A., Isfort R.J. Assessing the predictiveness of the Syrian Hamster Embryo Cell Transformation Assay for determining the rodent carcinogenic potential of single ring aromatic/nitroaromatic amine compounds. Toxicological Sciences 41, 198-197, 1998
60. Kiese, M. et al. (1968) The Absorption of Some Phenylenediamines Through the Skin of Dogs Toxicology and Applied Pharmacology 12, 495
61. Korossy, S. et al. (1969) Zur Revision des Allergenspektrums der Ungarn gebräuchlichen diagnostischen Standard-Reihe für Epikutantestung. Berufsdermatosen 17. 252-263
62. Krasteva, M. (1993) Study on the immuno-allergology of delayed contact dermatitis to paraphenylenediamine. PhD thesis, Medical Academy of Sofia, Bulgaria
63. Krause, W. et al. (1991) Comparative Pharmacokinetics of Abecamil in Rat Following Single and Multiple Intragastric Treatment and Continuous Administration via the Diet. Drug Metabolism and Disposition 19 (1), 29-35
64. Kvelland I. Hereditas 100: 295-8 (1984)
65. Kwalek J. C., Hallmark R. K., Andrews A. W. JNCI 71: 293-8 (1983)
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
68. L'Oréal, Aulnay-sous-bois., France (1996) Etude comparative de la réactivité in vitro de cinq colorants capillaires vis-à-vis de mitochondries isolées de muscles de Rat.. Report n°: TM 96/001
69. Lynde, C. W. et al. (1982) Screening patch tests in 4190 eczema patents 1972-81 Contact Dermatitis 8, 417-421
70. Maibach, H. I. et al. (1981) Percutaneous Penetration of Hair Dyes. J. Soc. Cosmet. Chem. 32, 223-229
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
72. Marks, J. G. et al. (1995) North American Contact Dermatitis Group Standard Tray Patch Test Results (1992 to 1994) American Journal of Contact Dermatitis 6, 160-165
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
74. Mascres, C. et al. (1974) A study of thr pathogenesis of muscle lesions induced by p-Phenylenediamine. L'Union Médicale du Canada 103, 672-677
75. Mathur, A.K. et al. (1990) Biochemical and histopathological changes following dermal exposure to paraphenylenediamine in Guinea Pigs. J Appl Toxicol 10(5), 383-386
76. Mathur, A.K. et al. (1992) Dermal toxicity of paraphenylenediamine. Biomedical and Environmental Sciences 5, 321-324
77. Mathur, A.K. et al (1995) Effects of dermal exposure to paraphenylenediamine in Guinea Pigs. J. Toxicol –Cut. & Ocular Toxicol. 14(3), 207-210
78. McFadden JP, Wakelin SH, Holloway DB, Basketter DA. (1998) Contact Dermatitis 39; 79-81
79. Mitchell, A.D. et al. (1988)Env. Mol. Mut. 1 (supl 13):37-101
80. Mitchell A. D., Rudd C. J., Caspary W. J. Env. Mol. Mut. 1 (suppl 13): 37-101 (1988).
81. Mohn. University of Leiden
82. Moneghini, C. L et al. (1980) La paraphénylènediamine dans les dermites allergiques de contact Med et Hyg. 38, 1577-1581

83. Müller, (1976) Untersuchung von 1,4-Diaminobenzol auf Mutagenität im Bakterientest. Batelle Institut, Frankfurt
84. Munday, R. et al (1989) Muscle necrosis by N-methylated p-Phebylenediamine in Rats: structure-activity relationships and correlation with free-radical production In Vitro Toxicology 57, 303-314
85. Modée, J. et al. (1962) A comparison of results of patch tests in 1951 and in 1961. Acta Dermato-venereol. 42, 280-289
86. National Cancer Institute (NCI) (1978) Bioassay of p-Phenylenediamine Dihydrochloride for Possible Carcinogenicity. DHEW Publication N° (NIH) 79-1730
87. Nethercott, J. K et al. (1991) Patch Testing With a Routine Screening Tray in North America, 1985 Through 1989: II. Gender and Response. Am. Journal of Contact Dermatitis 2, 130-134
88. Nielsen, N. H. et al. (1993) Sensibilisation de contact aux constituants des cosmétiques dans une population danoise non sé1ectionnée. La Glostrup allergy study, Danemark. Ann. Dermatol. Venereol. 120, 33-38
89. Nishi K., Nishioka H. Mut. Res. 104: 347-50 (1982)
90. Nishioka H. Mut Res 38: 345 (1976)
91. Nohmi T., Miyata R., Yoshikawa K., Ishidate M. J. J. Tox. Sci. 7: 61-9 (1982)
92. Oliveira, H. et al (July 1998) Reactivity to 35 standard allergens in a normal population. Fourth Congress of the European Society of Contact Dermatitis, Helsinki P-105
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
94. Rehani M.M. et al (1981). Distribution kinetics of 3H-labeled p-Phenylenediamine - A hair dye. Indian J Med Res 74, 129-134
95. Rehani M.M. et al (1979). Aqueous chamber kinetics of the 3H-labelled hair dye. Bull P.G.I. 13(4), 211-15
96. Reprotox (1978) Prüfung auf embryotoxische Wirkung an der Maus: p-Phenylendiamin
97. Rojanapo, W et al (1986) Carcinogenicity of an Oxidation Product of p-Phenylenediamine. Carcinogenesis 7 (12), 1997-2002
98. Rojanapo, W et al. (1986) Carcinogenicity of Hair Dyes used in Thailand. Thai Cancer Journal 12, 43-56
99. Romaguera, C. et al. (1980) Statistical and comparative study of 4600 patents tested in Barcelona 1973-1977. Contact Dermatitis 6, 309-315
100. Rudner, E. J. et al. (1975) The frequency of contact sensitivity in North America 1972-74. Contact Dermatitis 1; 277-280
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
103. Schutz, K. H. (1976) Comparable Studies of Sensitization of Different Hair Dye Ingredients. University of Hamburg
104. Segre, (1977) Rapport concernant les essais do toxicité aiguë chez la souris. Universita di Siena
105. Seiler, J.P. (1977) Inhibition of testicular DNA synthesis by chemical mutagens and carcinogens. Preliminary results in the validation of a novel short term test. Mutation Research 46(4), 305-10
106. Sertoli, A. et al. (1999) Epidemiological Survey of Contact Dermatitis in Italy (1984-1993) by GIRDCA (Gruppo Italiano Ricerca Dermatiti da Contatto e Ambientali). American Journal of Contact Dermatitis 10, 18-30
107. Shahin, M. et al. (1979) Personal communication, L'Oréial Laboratories
108. Shahin, M.M. et al. (1980) Studies on the mutagenicity of resorcinol and hydroxy-3-(p-amino)anilino-6,N-[(p-amino)phenol]benzoquinone-monoimine-1,4 in Salmonella typhimurium. Mutation Research 78(3), 2113-18
109. Sharma, P. P. et al (1990) Dose-dependent Pharmacokinetics and Teratogenic Activity of Topical Retinoids. Toxicologist 10 (1), 237
110. Shelby, Stasiewicz (1984)

111. Sir Hashim, M. et al. (1992) Poisoning from henna dye and para-phenylenediamine mixtures in children in Khartoum. Annals of Tropical Paediatrics 12, 3-6
112. Schnuch A, Geier J, Uter PJ et al. (1997) National rates and regional differences in sensitisation to allergens of the standard series. Contact Dermatitis 37: 200-209
113. Soler-Niedziela, L. et al. (1991) Studies on three structurally related phenylenediamines with the mouse micronucleus assay system. Mutation Research 259, 43-48
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. Stenbeck, F. G. et al. (1977) Noncarcinogenicity of Hair Dyes: Lifetime Percutaneous Applications in Mice and Rabbits. Fd. Cosmet. Toxicol. 15, 601-606
116. Storrs, F. J. et al. (1989) Prevalence and relevance of allergic reactions in patients patch tested in North America - 1984 to 1985. J. Am. Acad. Dermatol. 20,1038-1045
117. Thompson C. Z., Hill L. E., Epp J. K., Probst G. S. Environ. Mut. 15: 803-11 (1983)
118. Topham, J.C. (1980). The detection of carcinogen-induced sperm-head abnormalities in Mice. Mutation Research 69(1), 149-55
119. Tosti A, Pazzaglia M, Betazzoni M. (2000) Contact allergy from temporary tattoos. Arch Dermatol 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)
122. Uter, W. et al. (1998) Epidemiology of contact dermatitis. The information network of Departments of Dermatology (IVDK) in Germany. Eur. J. Dermatol 1, 36-40
123. Veien, N. K. et al. (1992) Patch Test Results From a Private Dermatologic Practice for Two Periods of 5 Years With a 10-Year Interval. American Journal of Contact Dermatitis 4,189-192
124. Venitt S., Searle C. E. INSERM 52: 263-72 (1975)
125. Wakelin SH, Creamer D, Rycroft RJG, White IR, McFadden JP. (1998) Contact dermatitis from paraphenylenediamine used as a skin paint. Contact Dermatitis 39: 92-93
126. Warbrick EV, Dearman RJ, Basketter DA, Kimber I. (1999) J Appl Toxicol 19; 255-260,
127. Watanabe T., Hirayama T., Fukui S. Mut. Res. 245: 15-22 (1990)
128. Williams, G.M. et al. (1982) Reliability of the hepatocyte primary culture/DNA repair test in testing of coded carcinogens and noncarcinogens. Mutation Research 97(5):359-70
129 Wolfram, L. J. et al. (1985) Percutaneous Penetration of Hair Dyes. Arch Dermatol Res 277, 235-241
130. Yabe, K et al. (1991) An Experimental Rhabdomyolysis due to Paraphenylenediamine Contained in Hair Dyes. Res Pract Forens Med 34, 109-115
131. Yabe, K. et al. (1992) The effect of a p-Phenylenediamine containing hair dye on the Ca2+ mobilization in the chemically skinned skeletal muscle of the Rat. Nippon Hoigaki Zasshi 46(2), 132-140

Submission IV, 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 noncancer risk assessment. An analysis of the default uncertainty factor. *Regulatory Toxicol. Pharmacol.* 27, 3-20, 1998

Supplementary data, December 2005

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

8. MDS Pharma, L'Arbresle, France. P-phenylenediamine – Embryotoxicity study by the oral route (gavage) in the rat (Segment II). Draft report, Study No. AA29083, December 2005
9. MDS Pharma, L'Arbresle, France. P-phenylenediamine - Analytical method validation to determine test item concentrations in formulations. Draft report, Study No. AA29403, November 2005

Submission V, 2011

References in italics are not submitted as full reports in the present dossier. Some of these reports were already provided within previous submission dossiers.

1. *Allais, L. (2005). p-Phenylenediamine – embryo toxicity study by the oral route (gavage) in the rat (Segment II). MDS Pharma Services, L'Arbresle, France, Study No. AA29083, 29 December, 2005*
2. Appleqvist T. Pharmacokinetics and mass balance of radioactivity in Sprague Dawley rats following single oral administration of [14C]-PPD by oral gavage. CIT, Evreux, France, Study No. 26336 PAR, 3 January 2005
3. Appleqvist T. Pharmacokinetics and mass balance of radioactivity in Sprague Dawley rats following single administration of [14C]-PPD by oral gavage. CIT, Evreux, France, Study No. 27160 PAR, 3 January 2005.
4. *Dorn, J.L.C.M., Walton, K., Renwick A.G. (2005). Human variability in xenobiotic metabolism and pathway-related uncertainty factors for chemical risk assessment: a review. Food Chem. Toxicol. 43, 203-216*
5. *Dressler, W.E., Appelqvis T. (2006). Plasma/blood pharmacokinetics and metabolism after dermal exposure to para-aminophenol and para-phenylenediamine. Food Chem. Toxicol. 44, 371-379*
6. *FDA, 2005. US Food and Drug Administration, Center for Drug Evaluation and Research, CDER. Guidance for Industry, Estimation the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult HealthyVolunteers. Available at: http://www.fda.gov*
7. *Garrigue, J.L., Ballantyne, M., Kumaravel, T., Lloyd, M., Nohynek, G.J., Kirkland, D., Toutain H. (2006). In vitro genotoxicity of para-phenylenediamine and its N-monoacetyl and N,N'-diacetyl metabolites. Mut. Res. 608, 58-71*
8. Hill, R.E. (1995). Paraphenylenediamine – 13-week oral (gavage) toxicity study in the rat. Toxicol Laboratories Limited, Ledbury, UK, Study No. LRL/44/94, 24 April, 1995
9. *Hueber-Becker F, Nohynek GJ, Meuling WJA, Benech-Kieffer F and Toutain H, 2004. Human systemic exposure to a [14C]-paraphenylenediamine-containing oxidative hair dye and correlation with in vitro percutaneous absorption in human and pig skin. Food Chemical Toxicology 42, 1227-1236.*
10. *Imaida, K. Ishihara, Y., Nishio, O., Nakanishi, K., Ito, N. (1983) Carcinogenicity and toxicity tests on p-phenyleneidiamine in F344 rats. Toxicol. Lett. 16:259-269*
11. *Industry (2009). Industry Submission on Reaction Products, Submitted September 2009, revised February 2010*
12. *IPCS (2005). International Program on Chemical Safety. Chemical-specific adjustment factors for inter-species differences and human variability. World Health Organization, Geneva, Switzerland*
13. Kawakubo, Y., Merck, H.F., Al Masaoudi, T., Sieben, S. and Bloemeke, B. (2000). N-acetylation of paraphenylenediamine in human skin and keratinocytes. J. Pharmacol. Exp. Therapeutics 292 (1): 150-155
14. Meuling, W.J.A. and de Bie, A.Th.H.J. (2009). Consumer exposure to an oxidative hair dye. A [^{14}C]-PPD labelled mass balance study. TNO Study code 7852. 1 September, 2009
15. Meuling, W.J.A. and de Bie, A.Th.H.J. (2010). Consumer exposure to an oxidative hair dye. A [^{14}C]-PPD labelled mass balance study. TNO Study code 6427, 30 March, 2010, amended 10 December, 2010

16. *Nohynek, G.J., Skare, J.A., Meuling, W.J.A.., Hein, D.W., deBie, A.T.H.J., Toutain, H. (2004). Urinary acetylated metabolites and N-acetyltransferase-2 genotype in human subjects treated with a para-phenylenediamine-containing oxidative hair dye. Food Chem. Toxicol. 42: 1885-1891*
17. *Nohynek, G.J., Duche, D., Garrigue, A., Meunier, P.A., Toutain, H., Leclaire, J. (2005). Under the skin: biotransformation of para-aminophenol and para-phenylenediamine in reconstructed human skin and human hepatocytes. Toxicol. Lett. 158, 196-212*
18. Prentice, D.E. (2010). A review of the results of the "Paraphenylenediamine (PPD) 13 week oral (gavage) toxicity study in the rat (LRL/44/94)". 27 January, 2010
19. Renwick, A.G. (2010). The safety of *p*-phenylenediamine as a constituent of oxidative hair dyes. An expert opinion by A.G. Renwick OBE, Emeritus Professor, School of Medicine, University of Southampton, UK
20. *Rojanapo, W., Kupradinun, P., Tepsuwan, A., Chutimataewin, S., Tanyakaset, M. (1986a). Carcinogenicity of an oxidation product of p-phenylenediamine. Carcinogenesis 7 (12), 1997-2002*
21. *Rojanapo, W., Kupradinum, P., Tepsuwan, A., Chutimataewin, S., and Tanyakset M. (1986b). Carcinogenicity of hair dyes used in Thailand. Thai Cancer J. 12, 43-56*
22. *Ryle, P.R. and Prentice, D.E. (2005). p-Phenylenediamine (PPD): Expert Opinion on Carcinogenic Potential, 14 June, 2005*
23. *SCCS (2010). Opinion on reaction products of oxidative hair dye ingredients formed during hair dyeing processes, SCCS/1311/10 adopted at SCCS 8th plenary meeting of 21 September 2010*
24. *SCCS (2010).The SCCS'S Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation 7th Revision, adopted at SCCS 9th plenary meeting of 14 December 2010*
25. *Sellers RS, Morton D, Michael B, Roome N, Johnson JK, Yano BL, Perry R, Schafer K (2007). Society of Toxicologic Pathology position paper: organ weight recommendations for toxicology studies. Toxicol Pathol, 35(5): 751-755*
26. *Williams, G.M. and Iatropoulos, M.J. (2002). Alteration of liver cell function and proliferation: distinction between adaptation and toxicity. Toxicol. Pathol. 30 (1), 41-53*

Additional references

A. Burnett C, Jacobs MM, Seppala A and Shubik P. (1980) Evaluation of the Toxicity and Carcinogenicity of Hair Dyes. J. Toxicol. Environ. Health 6: 247-257.
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. (2006) Severe allergic hair dye reactions in 8 children. Contact Dermatitis 54: 87-91.
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. 608: 58-71
H. Munday R, Manns E, Fowke EA, Hoggard GK (1990). Structure-activity relationships in the myotoxicity of ring-methylated p-phenylenediamins in rats and correlation with autoxidation rates in vitro. Chemical-Biological Interactions. 76: 31-45
I. Munday R, Manns E (1999). Muscle necrosis in rats induced by 2-methoxy-p-phenylenediamine. Food and Chemical Toxicology 37: 561-564

J. Bhargava P, Matthew P (2007). Hair dye poisoning. Journal of the Association of Physicians of India 55: 871-872

K. Singh N, Jatav OP, Gupta RK, Tailor MK (2008). Myocardial damage in hair dye poisoning - An uncommon presentation. Journal of the Association of Physicians of India. 56: 463-464

L. Chrispal A, Begum A, Ramya I, Zachariah A (2010). Hair dye poisoning--an emerging problem in the tropics: an experience from a tertiary care hospital in South India. Tropical Doctor. 40: 100-103

M. Sokolowski A, 2009; Salmonella typhimurium Reverse Mutation Assay with 2-[(4-aminophenyl)amino]-4-[(aminophenyl)imino]-1(4H)-naphtalenone(A007/A017/A007), Harlan RCC Study Number 1230401, Roßdorf/Germany, August 2009

N. Wollny H-E., 2009: Gene Mutation Assay in Chinese Hamster V79 Cells *in vitro* (V79/HPRT) with 2-[(4-aminophenyl)amino]-4-[(aminophenyl)imino]-1(4H)-naphtalenone (A007/A017/A007), Harlan-CCR Study Number 1230402, Roßdorf/Germany, August 2009

O. Gudi R., 2009: In Vitro Human Lymphocyte Micronucleus Assay with A007-A017-A007, Study Number AC22XW.348.BTL, Bioreliance, Rockville/USA, April 2009

P. Honarvar N., 2009: In vivo Unscheduled DNA Synthesis in Rat Hepatocytes with 2-[(4-aminophenyl)amino]-4-[(aminophenyl)imino]-1(4H)-naphtalenone (A007-A017-A007), Harlan-CCR Study Number 1243800, Roßdorf/Germany, August 2009

Q. Thyssen JP, White JML (2008) Epidemiological data on consumer allergy to p-phenylenediamine. Contact Dermatitis 59: 327–343

R. Hillen U, Dickel H, Löffler H, Pfützner W, Mahler V, Becker D, Brasch J, Worm M, Fuchs T,. John SM, Geier J (2011) Late reactions to patch test preparations with reduced concentrations of p-phenylenediamine: a multicentre investigation of the German Contact Dermatitis Research Group. Contact Dermatitis 64: 196–202

S. Fukunaga T, Kawagoe R, Hozumi H, Kanzaki T (1996) Contact anaphylaxis due to para-phenylenediamine. Contact Dermatitis 1996: 35: 185-186

T. Goldberg BJ, Herman FF, Hirata I (1987). Systemic anaphylaxis due to an oxidation product of p-phenylenediamine in a hair dye. Annals of Allergy. 1987: 58: 205-208)

U. Mavroleon G, Begishvili B, Frew AJ (1998) Anaphylaxis to hair dye: a case report. Correspondence in Clinical and Experimental Allergy. 1998: 28: 121-122

V. Pasche-Koo F, French L, Piletta-zanin R-A., Hauser C. (1988) Contact urticaria and shock to hair dye. Allergy. 1998: 53:904-905

W. Belton AL, Chira T (1997) Fatal anaphylactic reaction to hair dye. The American Journal of Forensic Medicine and Pathology. 1997: 18(3): 290-292

X. Wong GS, King CM. (2003) Immediate type hypersensitivity and allergic contact dermatitis to p-phenylenediame in hair dye. Contact Dermatitis 48; 166.

Y. Manuj Kr. Bharali & Karabi Dutta (2011) Testicular toxicity of paraphenylenediamine after subchronic topical application in rat. International Journal of Environmental Health Research, DOI:10.1080/09603123.2011.634388

Z. 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)