Scientific Committee on Consumer Safety

SCCS

OPINION ON

Hydroxypropyl p-phenylenediamine and its dihydrochloride salt (A165)

The SCCS adopted the final Opinion
at its plenary meeting on 30-31 October 2019

ACKNOWLEDGMENTS

SCCS members listed below are acknowledged for their valuable contribution to the finalisation of this Opinion.

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http://ec.europa.eu/health/scientific_committees/experts/declarations/sccs_en.htm

This Opinion has been subject to a commenting period of a minimum eight weeks after its initial publication (from 25 June 2019 until 09 September 2019).
Comments received during this time period were considered by the SCCS. For this Opinion, comments received resulted in the following main changes: the NOAEL for maternal and developmental toxicity of hydroxypropyl p-phenylenediamine HCl was changed and set at 30 mg/kg/day. The new information provided by the Applicant resulted in revisions in SCCS comments in sections 3.1.4, 3.1.5, 3.1.6, 3.1.9, 3.3.5 and 3.3.8.

1. ABSTRACT

The SCCS concludes the following:

1. *In light of the data provided, does the SCCS consider Hydroxypropyl p-phenylenediamine and its dihydrochloride salt (A165), safe when used in oxidative hair colouring products up to a maximum on-head concentration of 2 %?*

   In light of the data provided, the SCCS considers that hydroxypropyl p-phenylenediamine and its dihydrochloride salt are not safe when used in oxidative hair colouring products due to potential genotoxicity.

2. *Does the SCCS have any further scientific concerns with regard to the use of Hydroxypropyl p-phenylenediamine and its dihydrochloride salt (A165) in cosmetic products?*

   A mild to moderate eye irritation potential of the test item cannot be excluded. Hydroxypropyl p-phenylenediamine 2HCl is a moderate skin sensitiser based on animal data.

Keywords: SCCS, scientific opinion, Hydroxypropyl p-phenylenediamine and its dihydrochloride salt (A165), Regulation 1223/2009, CAS base 73793-79-0 and salt 1928659-47-5, SCCS/1608/19

Opinion to be cited as: SCCS (Scientific Committee on Consumer Safety), Opinion on Hydroxypropyl p-phenylenediamine and its dihydrochloride salt (A165), preliminary version of 20-21 June 2019, final version of 30-31 October 2019, SCCS/1608/19

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Two 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) and the Scientific Committee on Health, Environmental and Emerging Risks (SCHEER) and are made up of independent 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.).

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2. MANDATE FROM THE EUROPEAN COMMISSION

Background

Submission I on the hair dye Hydroxypropyl p-phenylenediamine and its dihydrochloride salt (A165), with the chemical name 3-(2,5-diaminophenyl)propan-1-ol (CAS 73793-79-0) and 3-(2,5-diaminophenyl)propan-1-ol-hydrochloride (CAS 1928659-47-5), was transmitted by Cosmetics Europe in July 2016.
The ingredients Hydroxypropyl p-phenylenediamine and its dihydrochloride salt (A165) are intended to be used in oxidative hair colouring products up to a maximum on-head concentration of 2 %.

Terms of reference

1. *In light of the data provided, does the SCCS consider Hydroxypropyl p-phenylenediamine and its dihydrochloride salt (A165), safe when used in oxidative hair colouring products up to a maximum on-head concentration of 2 %?*

2. *Does the SCCS have any further scientific concerns with regard to the use of Hydroxypropyl p-phenylenediamine and its dihydrochloride salt (A165) in cosmetic products?*

3. OPINION

3.1 Chemical and Physical Specifications

3.1.1 Chemical identity

3.1.1.1 Primary name and/or INCI name

Hydroxypropyl p-phenylenediamine (base)
Hydroxypropyl p-phenylenediamine 2HCl (dihydrochloride salt)

SCCS comment
The salt form of the test substance is a dihydrochloride (2HCl). SCCS noted that in the different study reports the SCCS received, the salt form was called Hydroxypropyl p-phenylenediamine HCl. Although this was not correctly named salt, the SCCS decided not to change this.

3.1.1.2 Chemical names

Base
3-(2,5-diaminophenyl)propan-1-ol
2,5-diaminobenzenepropanol
2-(3-hydroxypropyl)-p-phenylenediamine

Ref:
https://pubchem.ncbi.nlm.nih.gov/compound/15907303#section=Names-and-Identifiers
http://www.abichem.com/pro_result/?id=2518565

Dihydrochloride salt
3-(2,5-diaminophenyl)propan-1-ol-hydrochloride

3.1.1.3 Trade names and abbreviations

IMEXINE® OBN (*base)*
IMEXINE® OBK (*dihydrochloride salt)*

Other codes*:
R0025521A *(base)*
R0025521B *(dihydrochloride salt)*

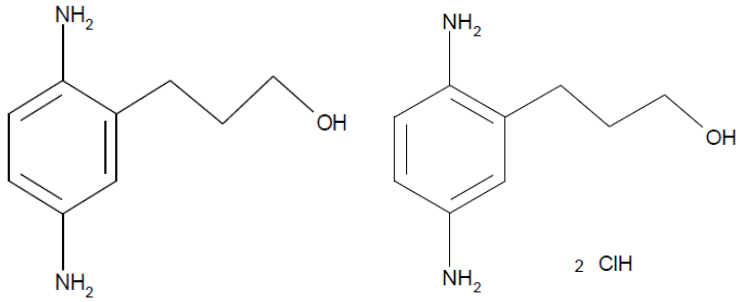
** codes used in the analytical file*

3.1.1.4 CAS / EC number

	Base	*Dihydrochloride salt*
CAS:	73793-79-0	1928659-47-5
EC:	/	/

3.1.1.5 Structural Formula

Base *Dihydrochloride salt*



3.1.1.6 Purity, composition and batch codes

	Base	*Dihydrochloride salt*
Formula	$C_9H_{14}N_2O$	$C_9H_{14}N_2O \bullet 2HCl$

3.1.2 Physical form

	Base	*Dihydrochloride salt*
Physical form:	Light pink to light purple powder	Pink beige powder

3.1.3 Molecular weight

	Base	*Dihydrochloride salt*
Molecular weight:	166.22 g/mol	239.14 g/mol

3.1.4 Purity, composition, and substance codes

Base:
The analytical study of R0025521A was performed on two batches:
Batch 005 D 001
Batch 001 L 002

Dihydrochloride salt:
The analytical study of R0025521B was performed on three batches:
Batch 004 P 001
Batch 006 L 002
Batch 006 L 003

The separation was achieved by reversed phase LC equipped with a photodiode array detector 2998.
All samples and eluents were filtered through a 0.2µm membrane filter (GHP) prior to use.
In the used HPLC chromatographic conditions, the expected compound is mainly detected in all tested batches:

HPLC relative purity*: >95 % (Area % without response factor, UV detection), process: wavelength λ = 210nm
* Irrespective of residual solvents, salts and other non-detectable products

All studies with hydroxypropyl p-phenylenediamine 2HCl and hydroxypropyl p-phenylenediamine submitted in the dossier were conducted using test batches 004 P 001 and 001 L002, respectively, which were well characterized analytically. The purity titres of batches 004 P 001 and 001 L002 were 97% and 101%, respectively. Other batches from each the base form and the dihydrochloride salt were characterized analytically as described in the table below.
Batch CFQ41882 of [^{14}C]-R0025521B (97% radiochemical pure) was used for the *in vitro* skin absorption study.

The titres of all tested batches are above or equal to 97% (w/w)
The titre of R0025521B 004 P 001 was estimated at 97% (w/w)
The titre of all other batches was determined against R0025521B 004 P 001 considered as 97 % pure.

Table 1: Comparative table of the analytical profiles of the different batches

	Hydroxypropyl p-phenylenediamine HCl			**Hydroxypropyl p-phenylenediamine**	
	004 P 001	**006 L 002**	**006 L 003**	**005 D001**	**001 L 002**
Appearance	Light beige powder	Light beige powder	Light beige powder	Light purple powder	Light pink powder
IR Spectrum	In accordance with the proposed structure			In accordance with the proposed structure	
UV-vis. Spectrum	Compatible with the proposed structure			Compatible with the proposed structure	Not carried out
Mass spectrum	Compatible with the proposed structure			Compatible with the proposed structure	
^{1}H NMR Spectrum	In accordance with the proposed structure			In accordance with the proposed structure	
Titer *(%-w/w)* **HPLC**	97 (Estimated titer***)	101.2±1.6[(1)]	99.8±1.4[(1)]	100.7±0.6[(1)]	101.0±0.8[(1)]
H.P.L.C. Profile UV Purity *(%)* [(2)]	> 95			>99	

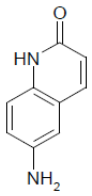
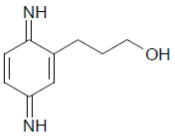
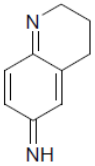
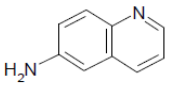
[(1)] *Titre determined against 3-(2,5-diaminophenyl)propan-1-ol dihydrochloride (R0025521B) batch 004 P 001 reference standard considered as pure (97% w/w).*
[(2)] UV detection *: UV purity* - Area %, without response factor.
Irrespective of residual solvents, salts and other non-detectable products

3.1.5 Impurities / accompanying contaminants

Potential impurities for R0025521 A/B can be starting material, synthesis intermediaries, by products or residual solvents.

Structural identification of unknown impurities:
Four impurities were detected in the various batches at a level greater than 0.1% (except impurity N°1). These impurities have been analysed by HPLC/DAD/ESI-MS and their structures are presented in Table 2.

Table 2: Structural formulas of the four impurities detected

Impurity number and description	**Structural formula**
Impurity Nº1: detected at *m/z* 161.1 in ESI(+); corresponds to a compound having a $C_9H_8N_2O$ empirical formula and a molecular weight of 160.1 g/mole. **6-Aminoquinolin-2-ol**	 Exact Mass =160.06366 Molecular Formula =C9H8N2C
Impurity N°2: detected at m/z 165.2 in ESI(+);corresponds to a compound having a $C_9H_{12}N_2O$ empirical formula and the molecular weight of 164.1 g/mole. Structural hypothesis: **Quinone di-imine of R0025521**	 Exact Mass =164.09496 Molecular Formula =C9H12N2C
Impurity N°3: detected at m/z 147.1 in ESI(+);corresponds to a compound having a $C_9H_{10}N_2$ empirical formula and a molecular weight of 146.1 g/mole. Structural hypothesis: **de-hydro form of R0024700A**.	 Exact Mass =146.08440 Molecular Formula =C9H10N2
Impurity N°4: detected at m/z 145.1 in ESI(+);corresponds to a compound having a $C_9H_8N_2$ empirical formula and a molecular mass of 144.1 g/mole. Structural hypothesis: **Quinolin-6-amine**	 Exact Mass =144.06875 Molecular Formula =C9H8N2

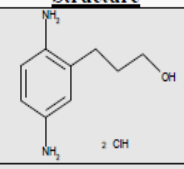
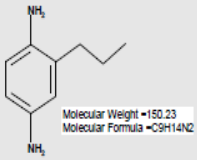
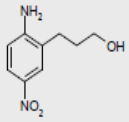
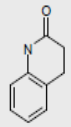
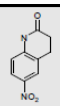
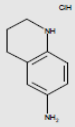
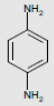
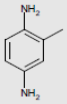
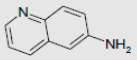
Some other impurities were detected in small quantities in all other tested batches and are presented in Table 3. The chemical structures of all the impurities are presented in Table 4.

Table 3: Other impurities

Impurities	R0025521B (dihydrochloride)			R0025521A (base)	
	004 P001	006 L002	006 L003	005 D001	001 L002
	% area (λ= 230 -700nm)				
9.3min (Imp.1)	ND	ND	ND	0.05	ND
11min (Imp.5 R0080941A)	ND	ND	0.01	0.02	ND
12.8min (Imp.2)	0.08	0.11	0.11	0.05	0.08
18min (Imp. 3)	0.19	0.06	0.07	0.04	0.06
Imp 6 R0024700C	Specific method was developed				
19.1min (Imp. 4 R0066611A)	ND	0.05	0.06	0.24	0.01
19.8min (Imp. 7 R0025495A)	0.59	ND	ND	Not generated in this process	
22.2min (Imp. 8 R0078355A)	ND	ND	ND	ND	ND
22.5min (Imp. 9 R0078106A)	ND	ND	ND	ND	ND
23.0min (Imp. 10 R0078356A)	ND	ND	ND	ND	ND

In green: the impurities to be quantified with a level greater than 0.1% (%area)
In red: specific method was developed for R0024700C
An analytical approach for the elucidation of the impurities with a level greater than 0.1% (%area) was performed.

Table 4: Impurities for R002551B

Code Chloe	Structure
R0025521B	
R0025495A	 Molecular Weight =150.23 Molecular Formula =C9H14N2
R0078106A	 Molecular Weight =196.21 Molecular Formula =C9H12N2O
R0078355A	 Molecular Weight =147.18 Molecular Formula =C9H9NO
R0078356A	 Molecular Weight =192.18 Molecular Formula =C9H8N2O3
R0024700C	 Molecular Weight =148.21 36.46 Molecular Formula =C9H12N2 . HCl
R0011102A/B	 Molecular Weight =108.14 Molecular Formula =C6H8N2
R0011103A	 Molecular Weight =122.17 Molecular Formula =C7H10N2
R0066511A	 Molecular Weight =144.18 Molecular Formula =C9H8N2

According to the analytical data obtained from the analysis of three batches for R0025521B (dihydrochloride) and two batches for R0025521A (base), both compounds seem to contain similar impurities. 2-propylbenzene-1,4-diamine dihydrochloride (R0025495A), was detected only in R0025521B Batch 004 P 001: 0.8% (w/w).

R0024700C content in:
- R0025521B (base) 005 D 001: < 0.1% (% w/w) Detected
- R0025521B (base) 001 L 002: < 0.1% (% w/w) Detected
- R0025521B (dihydrochloride) 004 P 001: < 0.1% (% w/w) Detected
- R0025521B (dihydrochloride) 006 L 002: < 0.1% (% w/w) Detected
- R0025521B (dihydrochloride) 006 L 003: < 0.1% (% w/w) Detected

Other impurities are detected. The relative % areas are given in Table 5.

Table 5: Impurities of the test substances in different batches

	Hydroxypropyl p-phenylenediamine HCl			Hydroxypropyl p-phenylenediamine	
	004 P 001	006 L 002	006 L 003	005 D001	001 L 002
Impurities H.P.L.C					
R0025495A *(%-w/w)* [3]	0.8	Not present in the process			
R0024700A *(µg/g)* [4]	<1000 (D**)	<1000 (D**)	<1000 (D**)	<1000 (D**)	<1000 (D**)
R0011102A [5] *(µg/g)*	<40µg/g (D**)	<40µg/g (ND*)	<40µg/ (ND*)	Not present in the process	
R0011103A [6] *(µg/g)*	<50µg/g (D**)	<50µg/g (ND*)	<50µg/g (ND*)	Not present in the process	
R0066511A [7]	<0.1% (ND*) [2]	<0.1% (D**) [2]	<0.1% (D**) [2]	0.09±0.005 % w/w	<0.1% (D**) [2]

**ND: Not detected – **D: Detected*
****Estimated titer of R0025521B (dihydrochloride):*
[100-(Impurities+Solvent contents)]X UV area(%) of main peak (exact value)

[2] UV detection: UV purity - Area %, without response factor. Irrespective of residual solvents, salts and other non-detectable products λ = 230-700 nm
[3] 2-propylbenzene-1,4-diamine dihydrochloride against batch R0025495A 001 L 001 reference standard considered as pure (99.9% w/w)
[4] 1,2,3,4-tetrahydroquinolin-6-amine hydrochloride against batch R0024700C 001 L 001 reference standard considered as pure (69% w/w)
[5] 4-aminophenylamine dihydrochloride against batch R0011102A 000 L 139 reference standard considered as pure (99.8% w/w)
[6] 2-methylbenzene-1,4-diamine dihydrochloride against batch R0011103A 001 L 001 reference standard considered as pure (99.3% w/w)
[7] Quinoline -6 amine

Ref: Analytical file Annex 11, 2014

During the public consultation period the Applicant clarified that impurities R0025495A, R0011102A and R0011103A are present as dihydrochloride or as base forms according to the main compound studied (A165 salt form or base form, respectively). Regarding impurity R0024700A, it is under base form as it is present in the base form of A165. However, R0024700C which is present in the salt form of A165, is a hydrochloride and not a dihydrochloride.

Residual solvents
The residual solvents content for the base was evaluated by NMR as follows:
- R0025521A (base) Batch 005 D 001
  - Ethanol: 0.06 % (w/w) (# 0.002 M/Mole)
- R0025521A (base) Batch 001 L 002
  - Methanol < 0.005M/Mole

Data are on the residual solvents content for R0025521B (dihydrochloride) are presented in Table 6.

During the public consultation period, the Applicant has calculated in accordance to ICH guidelines Q3A (R2), that the reporting, identification and qualification thresholds for the impurities applying to A165 (associated with a maximum daily dose <2g/day) according to ICH guidelines are considered to be at the levels of 0.05%, 0.1% and 0.15%, respectively. Accordingly, the characterization (identification) of impurities below 0.1% would not be mandatory.

Table 6: Residual solvents

Lots	Ethanol (µg/g)	Ethyl Ether (µg/g)	Chloroethane (µg/g)	Methylethylether (µg/g)
006 L 002	<1000	<100	20	
006 L 003	<1000	<100	30	
004 P 001	2600	500	500	50

SCCS comment

- Impurities at content above 0.1% have been quantified using either external reference standards of the corresponding impurities or area normalization. In case of the known impurities where an external reference standard is used, the SCCS is of the opinion that a response factor should be applied for the quantitation.
- Justification should be provided for the filtration of the samples using 0.2µm syringe filters prior to the HPLC analysis, as this would have removed any insoluble impurities. It should be demonstrated that the main compound and all the impurities are fully dissolved in the dilution solvent prior to the HPLC analysis. The percentage recovery of the filtration procedure should be calculated by analysing samples without filtration and comparing the corresponding peak areas (of the test substance and the impurities) with those obtained after filtration. HPLC-PDA chromatograms and % content of all impurities of these samples analysed with and without filtration should be comparable in terms of % area and retention times.
- Table 3 contains an error. Impurity 4 is not R0066611A, but R0066511A.
- All impurities above 0.1% should be accurately quantified in every batch and kept at trace levels.

3.1.6 Solubility

Base:
R0025521A batch 005 D 001:
Water Milli Q: 0.5 to 1 g/mL (pH about 7.6)
Absolute Ethanol: 0.1 to 0.5 g/mL
DMSO: 0.1 to 0.5 g/mL
Corn Oil: < 0.1 mg/mL
* Data generated by PharmaPhysic on R0025521A (base) Batch 005 D 001

Dihydrochloride salt
R0025521B, batch number R0025521B 004 P 001
Preliminary solubility data indicated that R0025521B was soluble in water for irrigation (purified water) at a concentration of at least 50 mg/mL

Ref: Hobson, 2012.

Preliminary solubility data indicated that R0025521B was soluble in water for irrigation (purified water) at a concentration of at least 50.94 mg/mL.

Ref: Llyod, 2013

SCCS comment
Solubility data of both the base and dihydrochloride salt (test substances) in the dilution solvents used for the various studies, including the solvents used for peak purity evaluation and impurity testing, have not been provided. Solubility should be assessed according to OECD TG 105.
During the public consultation period, the Applicant has communicated to the SCCS that they will provide a solubility study in water according to OECD 105 for both the dihydrochloride and base forms in the forthcoming submission.

3.1.7 Partition coefficient (Log P_{ow})

Base:
No data provided

Dihydrochloride salt:
R0025521B batch 004 P 001
Log Pow Calculated: -0.70 code error 0 (ClogP, v5.2)
Log Pow Experimental: -0.60 code error 0.012 (determination by potentiometry)

3.1.8 Additional physicochemical specifications

Ionization constant
Base:
No data provided

Dihydrochloride salt:
R0025521B batch 004 P 001
Experimental pKa are 2.92 and 6.11 (25°C, ionic strength 0.15M) for a basic equilibria NH+/NH (GLpKa Sirius)
pH=2.0 (2% w/w in distilled water)

Ref: Maillet, 2012

UV-Vis spectra
Base:
The UV/Visabsorption, in the range 200 to 800 nm of a 0.0005g/100mL solution of R0025521A (base) in water exhibited three maxima at about 200 nm, 240 nm and 290 nm.

Table 7: UV-Vis spectra of R0025521A (base) Batch 005 D 001

	R0025521A (base) Batch 005 D 001 Concentration: # 0.0005% w/w in water
Absorbance (λ=200nm)	1.058
E1% - 1cm	2116
Absorbance (λ=240nm)	0.245
E1% - 1cm	490
Absorbance (λ=290nm)	0.062
E1% - 1cm	124

Dihydrochloride salt:
The ultra-violet/visible light absorption, in the range 190 to 700 nm of a 0.0004g/100mL solution of R0025521B (dihydrochloride) in water exhibited three maxima at 200nm, 240nm and 290nm.

Table 8: UV-Vis spectra of ROO25521B (dihydrochloride) batches 004 P001, 006 L002, 006 L003

	R0025521B (dihydrochloride) Batch 004 P 001	**R0025521B (dihydrochloride) Batch 006 L 002**	**R0025521B (dihydrochloride) Batch 006 L 003**
	Concentration: # 0.0004% w/w in water		
Absorbance (λ=200nm)	0.808	0.700	0.643
E1% - 1cm	2020	1750	1607.5
Absorbance (λ=240nm)	0.148	0.127	0.117
E1% - 1cm	370	317.5	292.5
Absorbance (λ=290nm)	0.034	0.028	0.025
E1% - 1cm	85	70	62.5

Heavy metals content was determined.

Ref: Analytical file, 2014

3.1.9 Homogeneity and Stability

Active Ingredient concentration and homogeneity in dose formulations
The analysis was performed to determine the active ingredient concentration and homogeneity of R0025521B in dose formulation using HPLC for JRF Study Number: 443-03-5651.

Ref: Gohel D, Volume II, 2014b

Stability
Solutions of the test substance prepared in water at concentrations 1, 3 and 10 mg/mL were found to be stable for up to 4 hour after preparation.

Ref: Gohel D, Volume I, 2014a.

Stability of the Test Item in the Vehicle
The stability of active ingredient in reverse osmosis water was determined prior to initiation of the study after validation of the analytical method (JRF Study No 228-2-13-5667). The stability of test item was determined at 0 and 4 hours at room temperature.

Ref: Patel, 2014

The HPLC analysis of the hair dye formulation performed following the dosing procedure and 24 hours post application was 102% and 97.8%, respectively, confirming that the formulation was stable for a 24-hour period.

Ref: Toner, 2014

Storage conditions
Base:
Batches: R0025521A 005D 001 and R0025521A 001 L 002
Storage conditions: Refrigerated at 4°C under inert gas, away from light and shielded from humidity. The compound is considered to be stable when stored in the appropriate aforementioned conditions.

Dihydrochloride salt:
Batches R0025521B 004 P001, R0025521B 006 L002 and R0025521B 006L 003
Storage conditions: Refrigerated at 4°C under inert gas, away from light and shielded from humidity. The compound is considered to be stable when stored in the appropriate aforementioned conditions.

Ref: Analytical File, 2014

The raw material (batch: R0025521B 004 P 001) was stored away from light and humidity, tightly closed, in a well ventilated laboratory, at room temperature protected from air (under inert gas). Analytical certificate version 2: According to the Applicant, this new analysis certificate is issued to check the stability of the compound. Storage conditions: Ambient temperature, under inert gas, away from light and shielded from humidity. The compound is considered to be stable when stored in the appropriate aforementioned conditions.

Ref: Amsellem, 2014

During the public consultation period the Applicant provided clarification on the storage conditions. The salt form of A165 (R0025521B batch 004 P 001) has been initially stored at room temperature (20-25°C) according to the certificate of analysis version 2 (edited in 2013) and version 3 (edited in 2015). Giving that this batch remained stable between 2013 and 2015, this confirms the stability of A165 when stored at room temperature. A stability study conducted in 2016 showed that A165 is stable at 4°C for more than two months Furthermore, the Applicant explored the stability of A165 under more stringent conditions like high temperature reaching 45°C., however degradation was observed at 45°C after a period of one month. Thus, as a precautionary measure, the Applicant recommends to store A165 at 4°C, and this was reported in the version 4 of the certificates of analysis in 2016.

SCCS comment
Detailed data on the stability of test substance in formulations should be provided by HPLC-PDA analysis over the wavelength range (200-400 nm).

3.2 Function and uses

The ingredients hydroxypropyl p-phenylenediamine and its dihydrochloride salt (A165) are intended to be used in oxidative hair colouring products at on-head concentration up to 2%.
According to the Applicant, hydroxypropyl p-phenylenediamine is a weak base, and as such may be formulated in the chemical form of a pure base, or as the salt of a simple inorganic counter-anion such as dihydrochloride. The salt form may be used in order to improve stability, solubility or to facilitate the formulation of the respective base. Choosing for a hair dye ingredient either the salt or free base form is linked to the ease and stability under storage conditions of these raw materials.
When formulated in finished oxidative hair coloring products, these two chemical forms of the hair dye ingredient A165 (free base and dihydrochloride salt) are quantitatively present as or converted to the free base. This is a consequence of the high pH value of oxidative hair coloring products (typically of about 9-10), which is significantly higher than the pKa values of hydroxypropyl p-phenylenediamine (2.9 and 6.1 for each of the two amine functions of A165). Accordingly, only hydroxypropyl p-phenylenediamine (free base) is present in oxidative hair coloring products containing the hair dye ingredient A165, whatever the initial form (salt or free base) of the ingredient used to formulate the product.

3.3 Toxicological evaluation

3.3.1 Acute toxicity

3.3.1.1 Acute oral toxicity

No acute oral toxicity studies were performed with the hydroxypropyl p-phenylenediamine dihydrochloride salt. Nevertheless, in the 14-day repeated oral toxicity study performed by oral gavage in rats with hydroxypropyl p-phenylenediamine 2HCl at dose levels of 100, 300 and 1000 mg/kg, mortality was observed in all rats (10/10) treated at 1000 mg/kg/day at day 1, 2 or 3 post dosing. One death (1/10) was noted at the mid dose level of 300 mg/kg/day at day 14 after dosing. Lung and liver congestion were noted at necropsy. The clinical signs observed included lethargy, tremor, salivation, lacrimation, chromodacryorrhea and prostration in the high dose group. Only mild salivation and lethargy were observed in the mid dose group. Accordingly, the median lethal dose (LD50) was considered to be between 300 and 1000 mg/kg, and hydroxypropyl p-phenylenediamine and its dihydrochloride salt were considered to be of moderate toxicity following single oral administration.

Ref.: Mehta, 2013

SCCS comment
The 14-day oral repeated dose toxicity study provides evidence that hydroxypropyl p-phenylenediamine 2HCl caused mortality at dose levels of 300 and 1000 mg/kg. This study indicates that the LD50 is above 300 mg/kg.

3.3.1.2 Acute dermal toxicity

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3.3.1.3 Acute inhalation toxicity

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3.3.1.4 Acute intraperitoneal toxicity

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3.3.2 Irritation and corrosivity

3.3.2.1 Skin irritation

***In vitro* Episkin^{SM} Skin Irritation Test**

Guideline:	Based on OECD 439, Test method validated by EU Reference Laboratory for alternatives to animal testing (EURL-ECVAM) (ESAC statement, 2007)
Test System:	Reconstructed human epidermis model Episkin, small model (0.38 cm^2)
Replicates:	3 different tissue batches
Test substance:	Hydroxypropyl p-phenylenediamine HCl
Batch:	R0025521B 004 P 001
Purity:	98%
Test item:	raw material diluted at 2.0% RM in water
Dose level:	2.0%
Treatment period:	15 minutes
Post-treatment incubation time:	42 ± 1 hour
Positive control:	10 µL of 50 mg/mL aqueous solution of Sodium Dodecyl Sulfate
Negative control:	10 µL of PBS+;
Solvent control:	10 µL of the water
Dead dermis negative control:	10 µL of PBS+ tested in triplicate on dead epidermis
Interaction with MTT:	Positive
Colouring of tissue:	Positive
GLP:	In compliance
Study period:	September 2013 – April 2014

The test item hydroxypropyl p-phenylenediamine HCl tested at the concentration of 2% in water was evaluated on 3 different batches of reconstructed human epidermis model. After preliminary tests (dilution and staining tests), the coloured test item was tested according to the specific colouring protocol.
Hydroxypropyl p-phenylenediamine HCl, negative control (PBS+), positive control (5% aqueous solution of Sodium Dodecyl Sulfate), solvent control (water), a dead epidermis negative control (PBS+) were tested in triplicate. Two additional negative controls (one dead and one alive tissue) which followed the same treatment as the negative control except the MTT incubation period were added. Six additional tissues (three alive and three dead tissues) were used and followed the same treatment with the test item as the other tissues (except for the MTT incubation period). These tissues as well as the additional negative control were used as additional specific controls in order to quantify the Non Specific Colour due to the colouring chemical interactions with the tissue. 10 µL of hydroxypropyl p-phenylenediamine HCl tested at the concentration of 2% and of different controls were applied onto the epidermis using a positive displacement pipette. After 15 minutes treatment period at room temperature tissues were rinsed with PBS+, and then epidermis were transferred in 2 ml/well of fresh maintenance medium and incubated for 42 hours ± 1 hour at 37°C.
At the end of the 42 hours ± 1 hour treatment period, each epidermis unit was transferred to 12-well plate containing a dye solution (MTT) except for the negative control and the test item-treated epidermis without MTT which were transferred into a 12-well plate containing fresh medium. Plates were incubated for 3 hours approximately at 37°C. At the end of the incubation period, a biopsy of the entire epidermis was taken. For all tissues with the test item, the superficial epidermis layer (containing most of the remaining colour) was removed and discarded. The epidermis was separated from the collagen matrix and both were transferred into a tube containing 500 µL of acidified isopropanol. Formazan crystals were extracted and stirred to homogenise the solution. 2 x 200 µL of each extract were transferred onto a 96-well plate and the optical density (OD) was measured at 570 nm versus acidified isopropanol.
IL-1α released in the culture medium was determined by a classic quantitative sandwich enzyme immunoassay technique. The optical density (OD values related to the IL-1 α amount) was measured at 450 nm. The test item is predicted to be non-irritant when mean viability value is above 50% and final IL-1α release is below 50 pg/mL. The test item is predicted to be irritant when mean viability value is lower than (≤) 50% or final IL-1α release is above (≥) 50 pg/mL.

Results
The mean viability value for hydroxypropyl p-phenylenediamine HCl at the concentration of 2% was 85.3% (SD 5.2%) and the final IL-1α release was 4 pg/mL.

Conclusion
Under the conditions of this study, the *in vitro* evaluation of acute skin irritation by using the reconstructed human epidermis Episkin[SM] model, suggests that hydroxypropyl p-phenylenediamine HCl when tested at the concentration of 2% in water was considered as potentially non-irritating.
Ref.: Amsellem, 2014

SCCS comment
Even though hydroxypropyl p-phenylenediamine 2HCl diluted to 2% w/w in water has a very low pH of 2, which may lead to corrosive effects, skin irritation was not detected in the *in vitro* Episkin study.

3.3.2.2 Mucous membrane irritation / Eye irritation

Bovine corneal opacity and permeability method (BCOP)

Guideline:	Modified version of OECD 437 (September 2009)
Test material:	Bovine cornea
Replicates:	6 cornea per condition
Test substance:	Hydroxypropyl p-phenylenediamine HCl
Batch:	R0025521B 004 P 001
Purity:	97.2%
Test item:	2% (w/w) in distilled water
Dose applied:	750µl
Treatment period:	30 minutes ± 5 minutes or 4 hours ± 10 minutes
Post-treatment incubation time	2 hours for 30 minutes incubation time, none for 4 hours incubation time
Positive control:	Cetyl Trimethylammonium Bromide (CTAB) in distilled water
Negative control:	Nutritive medium
GLP:	In compliance
Study period:	April – June 2012

Bovine eyes (from cattle aged at least 9 months) were collected from the slaughterhouse and prepared within 4 hours after collection. Eyes that were too big or cornea showing defects were discarded. After a 1 hour±10 minutes pre-incubation period at 32±1°C, a first basal opacity measurement of the fresh corneas was performed. Subsequently, 750 µl of the test item was applied onto the cornea for 30 minutes or 4 hours. After the 30 minutes treatment period, the cornea were rinsed and incubated for further 2 hours at 32±1 °C, after which opacity was measured again. In case of the 4 hour contact period, measurement of opacity was performed immediately after rinsing. Following the opacity readings, the permeability endpoint was measured. Hereto, the cornea were brought into contact via the anterior chamber of the corneal holder with 1ml of a 0.5% (w/v) sodium fluorescein solution in PBS^+ and incubated for 90±10 minutes at 32±1 °C. Medium from the posterior compartment of the corneal holder was sampled to measure the optical density at 490nm. The corneal score which is the combination of opacity and permeability was then calculated. Negative and positive control substances were tested according to the same experimental conditions.

Results
The score obtained for hydroxypropyl p-phenylenediamine HCl at the concentration of 2% (w/w) in water after 30-minutes and 4-hour contact was 2.3±1.9 and 3.5±6.3, respectively.

Conclusion
Under the conditions of this study, the *in vitro* evaluation of the acute ocular irritation potential of hydroxypropyl p-phenylenediamine HCl at a concentration of 2% in the Bovine Corneal Opacity and Permeability test, suggested that this ingredient is slightly irritant for the isolated bovine cornea after 30 minutes and 4 hours of contact.
Ref: Maillet, 2012

SCCS comment
The BCOP assay is an *in vitro* method that can be used to identify ocular corrosive and severe irritants, but not mild irritants. Even though hydroxypropyl p-phenylenediamine 2HCl diluted to 2% w/w in water has a very low pH of 2, which may lead to corrosive effects, the BCOP assay did not indicate severe eye irritation. This, however, does not exclude mild or moderate eye irritancy

potential. Under the conditions of this study, a mild to moderate eye irritation potential of the test item cannot be excluded.

3.3.3 Skin sensitisation

Local Lymph Node Assay (LLNA)

Guideline:	OECD 429, EC B.42
Species/strain:	Female CBA/J mice
Group size:	4 mice per group (main study), 2 mice per group (preliminary assay)
Test substance:	Hydroxypropyl p-phenylenediamine HCl
Batch:	R0025521B 004 P 001
Purity:	97.2% pure (relative purity was 98.6%)
Vehicle:	Propylene glycol
Concentration:	1, 2.5, 5, 10 or 25% (w/v)
Positive control:	alpha-hexylcinnamaldehyde (HCA) at 25% (v/v) in Propylene glycol.
GLP:	In compliance
Study period:	May – August, 2012

Animals were separated in groups (4 mice/group) consisting of five treated groups receiving hydroxypropyl p-phenylenediamine HCl at 1, 2.5, 5, 10 or 25% (w/v) in propylene glycol. Due to unsatisfactory solubility tests of hydroxypropyl p-phenylenediamine HCl in the first recommended vehicles, this vehicle was selected after a solubility study showing that 25% (w/v) hydroxypropyl p-phenylenediamine HCl was the maximal practicable concentration.
The test substance hydroxypropyl p-phenylenediamine 2HCl, propylene glycol or HCA were applied over the ears for three consecutive days. After 2 days of resting, mice received a single intravenous injection of tritiated methyl thymidine (3H-TdR). Lymph nodes draining the application sites (auricular nodes) were sampled, pooled per group, and the proliferation of lymphocytes was evaluated by measuring the incorporation of 3H-TdR. The values obtained were used to calculate stimulation indices (SI). The irritant potential of the test item was assessed by measuring ear thickness on days 0, 2 and 5.

Results
In the preliminary assay, treated mice showed no sign of toxicity at the dose concentration of 1.0%, 5.0%, 10.0% and 25% (w/v). There was no noteworthy increase observed in ear thickness measurement. There were lymphoproliferative responses with SI values of 1.72, 2.35, 3.53, 15.97 and 19.2 obtained at hydroxypropyl p-phenylenediamine HCl concentrations of 1%, 2.5%, 5%, 10% and 25%, respectively. The threshold value of 3 for positive results was exceeded at 5%, and the EC3 value was calculated to be 3.88%.

Conclusion
Under the conditions of this study, hydroxypropyl p-phenylenediamine HCl did induce delayed contact hypersensitivity. According to the EC3 value calculated (3.88%), hydroxypropyl p-phenylenediamine HCl was considered to have a moderate sensitising potential.

Ref: Verma, 2012

SCCS comment
The SCCS agrees with the Applicant that hydroxypropyl p-phenylenediamine 2HCl is a moderate skin sensitiser.
The Applicant stated that there was no noteworthy increase in ear thickness, but this is not supported by the provided data that show an increase in ear thickness in all exposed mice. In the high-dose group an increase of 20% in ear thickness was reported. In view of this, the SCCS considers hydroxypropyl p-phenylenediamine 2HCl as slightly irritant under the conditions of this study.

3.3.4 Dermal / percutaneous absorption

Guideline:	OECD 428
Species/strain:	Frozen human dermatomed skin (400 µm)
Membrane integrity:	Checked by electrical resistance, at least 10 kΩ
Replicates:	12 intact skin samples (4 donors)
Test substance:	Hydroxypropyl p-phenylenediamine HCl

Batch:	R0025521B 004 P 001 and R0025521B CFQ41882 of [^{14}C]
Purity:	99.3% (004 P 001); 97% (CFQ41882)
Test item:	Hair dye formulation containing a final on-head concentration of 2% hydroxypropyl p-phenylenediamine HCl
Dose applied:	20 mg/cm^2 of the test formulation (400 µg/cm2 of hydroxypropyl p-phenylenediamine HCl)
Exposure area:	2.54 cm^2
Exposure period:	30 minutes
Sampling period:	24 hours
Receptor fluid:	degassed phosphate buffered saline (PBS)
Solubility in receptor fluid:	100 g/L in water
Mass balance analysis:	100.20% ± 0.83% of the applied dose
Tape stripping:	Yes (20)
Method of Analysis:	Liquid scintillation counting (LSC)
GLP:	In compliance
Study period:	March – October, 2014

Skin samples were dermatomed (~400 µm in thickness) and mounted in diffusion cells, using PBS as the receptor fluid. Membrane integrity was determined by measurement of the penetration of tritiated water across the skin membrane. Membranes exhibiting absorption greater than 0.6% of the applied dose were regarded as having a lower integrity than normal and not used for exposure to the test materials. Twelve intact skin membranes (from four human donors) were used and skin was maintained at approximately 32°C.
A typical oxidative hair dye formulation containing a nominal 4% hydroxypropyl p-phenylenediamine HCl was mixed with peroxide developer (1:1, w/w) resulting in a concentration of 2% hydroxypropyl p-phenylenediamine HCl. About 20 mg/cm² of hydroxypropyl p-phenylenediamine HCl (corresponding to a nominal 400 µg/cm² of hydroxypropyl p-phenylenediamine HCl) were applied to the skin surface for 30 minutes to mimic in-use conditions. After this time period, the remaining formulation on the skin surface was removed using a standardized washing procedure, simulating use conditions. Twenty-four (24) hours after application, the percutaneous absorption of hydroxypropyl p-phenylenediamine HCl was estimated by measuring its concentration by liquid scintillation counting in the following compartments: skin washes, stratum corneum (isolated by tape strippings), living epidermis/dermis, unexposed skin and receptor fluid.

Results
All diffusion cells yielded data that could be analysed and the mean recovery rate was good at 100.2%. The HPLC analysis of the hair dye formulation performed following the dosing procedure and 24 hours post application was 102% and 97.8%, respectively, confirming that the formulation was stable for a 24-hour period. Most of the hydroxypropyl p-phenylenediamine HCl applied on the skin surface was removed with the skin wash at 30 minutes (95.98%). The mean amount of hydroxypropyl p-phenylenediamine HCl considered as systemically available was estimated as follows (sum of amounts measured in living epidermis/dermis and receptor fluid): 2.09 ± 1.61 µg-eq/cm^2 (0.54 ± 0.41% of the applied dose).

Conclusion
The results obtained in this study indicate that hydroxypropyl p-phenylenediamine HCl present at a maximum final on-head concentration of 2% in a typical oxidative hair dye formulation penetrated through human dermatomed skin at a very slow rate. The amount of hydroxypropyl p-phenylenediamine HCl considered as absorbed was estimated to be at most 2.09 ± 1.61 µg-eq/cm2 corresponding to 0.54 ± 0.41% of the applied dose.

Table 10: Summary of Hydroxypropyl p-phenylenediamine HCl distribution in the test system

	µg-eq/cm^2 Hydroxypropyl p-phenylenediamine HCl		% of applied dose	
	Mean	SD	Mean	SD
Total Dislodgeable dose	376.56	23.49	96.75	1.94
Unabsorbed dose	387.78	21.6	99.66	1.15
Absorbed dose	0.26	0.16	0.07	0.04
Dermal delivey	2.09	1.61	0.54	0.41
Mass balance	389.87	21.36	100.2	0.83

Unabsorbed dose = total dislodgeable dose + stratum corneum + unexposed skin.
Absorbed dose = receptor fluid + receptor rinse + receptor wash
Dermal delivery = epidermis + dermis = absorbed dose

Ref.: Toner, 2014

SCCS comment
Following the SCCS Notes of Guidance, the dermal absorption of hydroxypropyl p-phenylenediamine 2HCl was estimated to be 3.7 µg/cm^2 (mean + 1SD).

3.3.5 Repeated dose toxicity

3.3.5.1 Repeated Dose (14 days) oral toxicity

Guideline: /
Species/strain: Wistar rats
Group size: 5/sex/group
Test substance: Hydroxypropyl p-phenylenediamine HCl
Batch: R0025521B 004 P 001
Purity: 98.6%
Vehicle: Distilled water
Dose levels: 0, 100, 300 or 1000 mg/kg
Dose volume: 10 mL/kg
Route: Oral
Administration: Gavage
GLP: In compliance
Study period: May 2012 – June 2012

In the main study, rats (5/sex/group) received a 14-day repeated oral (gavage) dose of hydroxypropyl p-phenylenediamine HCl at 0, 100, 300 or 1000 mg/kg in distilled water at 10 mL/kg. Animals from hydroxypropyl p-phenylenediamine HCl or vehicle control groups were killed 24 hours after the last administration.

Results
Mortality was observed in the high and mid dose groups (10/10 and 1/10, respectively). Rats in the mid dose group (300 mg/kg) showed significant reductions in body weight gain compared to controls, from day 4 onwards in males and from day 8 onwards in females. Only an increase in relative weight of liver (relative to terminal body weight) and in the level of triglycerides was observed in female rats treated with the dose 100 mg/kg/day. Yellow to yellowish brown colored urine was also observed, confirming the systemic exposure to hydroxypropyl p-phenylenediamine HCl.

Conclusion
Thus, the dose level of 300 mg/kg can be considered a maximum tolerated dose.

Ref.: Mehta, 2013

SCCS comment
Statistically significant decreases in red blood cell count, hematocrit and prothrombin time were observed in females at 300 mg/kg bw/d. In both sexes, statistically significant increases in platelet counts were observed at the highest dose tested. In addition, a trend increase in absolute and relative liver and kidney organ weights was observed. These changes were significant at 300 mg/kg/day. Therefore, a NOAEL of 100 mg/kg/day Hydroxypropyl p-phenylenediamine 2HCl can be derived from this study.

3.3.5.2 Sub-chronic (90 days) toxicity (oral)

Guideline:	OECD TG 408
Species/strain:	Wistar rats
Group size:	10 animals/sex/group
Test substance:	Hydroxypropyl p-phenylenediamine HCl
Batch:	R0025521B 004 P 001
Purity:	97.2% (relative purity 98.6%)
Vehicle:	RO water
Dose levels:	0, 10, 30 or 100 mg/kg/day
Dose volume:	10 mL/kg
Route:	oral
Administration:	gavage
GLP:	in compliance
Study period:	14 December 2012 – 24 May, 2014 (the experimental phase of this study was conducted from December 2012 to 22 March 2013. The corresponding study report was completed in 2014).

The subchronic toxicity of hydroxypropyl p-phenylenediamine HCl was investigated in Wistar rats (10/sex/group) after daily oral gavage at 0, 10, 30 or 100 mg/kg/day in water (10 mL/kg) for 13 weeks. These dose levels were selected on the basis of the results of a preliminary 14-day study performed at 100, 300 and 1000 mg/kg/day, described in section 3.3.18.1). Evaluations and measurements included mortality checks, daily clinical observations, weekly body weight and food intake, ophthalmoscopy prior to dosing and at the end of the treatment period, neurotoxicological evaluation during week 12, haematology, blood clinical chemistry and urinalysis (week 13). At the end of treatment period, surviving animals were killed and subjected to macroscopic examination; selected organs were weighed, and a wide range of organs/tissues were preserved. Microscopic examination was performed for specified tissues/organs from control and high dose rats killed at the end of the dosing period, as well as for any gross anomaly.

Results
The chemical analysis of the dose formulations administered during the study showed that achieved concentrations were close to the intended values. The test item hydroxypropyl p-phenylenediamine HCl did not induce any relevant treatment-related changes with respect to survival, clinical signs, body weight, body weight gain, food consumption, ophthalmological examinations, neurological observations as well as coagulation and urine parameters evaluated. No toxicologically significant changes in hematology parameters were observed.
Statistically higher liver weight was observed in both males (both absolute and relative to terminal bodyweight) belonging to the high dose group when compared to the vehicle control group. In high dose females, the relative liver weights were statistically significantly ($p \leq 0.01$) increased (3.491 versus 2.972 in controls). In the absence of microscopic lesions of pathological significance, these liver effects are considered as an adaptive response to the test item and of little relevance to man.
Statistically significant higher alanine transaminase (ALT), Aspartate Aminotransferase (AST), total bilirubin, cholesterol and GGT values were observed in females from the high dose group when compared to the vehicle control.

- ALT ($p \leq 0.05$): 44.61 IU/L in high dose females compared to 36.67 IU/L in controls
- AST ($p \leq 0.01$): 287.95 IU/L in high dose females compared to 104.3 IU/L in controls

- Total Bilirubin (p≤0.05): 3.98 µmol/L in high dose females compared to 3.09 µmol/L in controls
- Cholesterol (p≤0.01): 68.9 mg/dL in high dose females compared to 50.45 µmol/L in controls
- GGT (p≤0.05): 0.38 IU/L in high dose females compared to 0.02 IU/L in controls

When compared to controls, the statistically significant higher level of AST correlated with a statistically significant higher absolute (in males) and relative (in males and females) liver organ weights were observed at the highest dose level, without any associated microscopic changes. The increased values of AST were considered as test-item related, whereas all other changes (ALT, total bilirubin, cholesterol and GGT) were considered incidental and unrelated to treatment due to their low magnitude. Statistically significant lower absolute heart weight was observed in females from the high dose group, while significant higher kidney weight was observed in the males from the same dose group. These values were within the historical control range values and were considered unrelated to the treatment. No other test item-related changes were detected regarding absolute and relative organ weights, gross and microscopic examination.

Conclusion
Thus, under the conditions of the study, the NOAEL (No Observed Adverse Effect Level) of this 90-day oral toxicity study on hydroxypropyl p-phenylenediamine HCl was 100 mg/kg/day, and the No Observed Effect Level (NOEL) was 30 mg/kg/day in Wistar rats.

Ref.: Gohel, 2014a, b

SCCS comment
The SCCS agrees with the Applicant that the increase in reticulocyte count and the hematological effects are probably not biologically and toxicologically relevant. The SCCS however does consider the significant effects in some clinical chemistry parameters as well as the statistically significant increase in relative liver weights of females at the high dose as adverse and of toxicological relevance. Hall et al (2012) suggest that in the absence of histological changes a weight-of evidence approach should be used by considering biologically significant and consistent increase in at least two further liver parameters. Since both ALT and AST levels were statistically significantly increased in the high dose females compared to controls, the SCCS considers the liver effects as adverse, resulting in a NOAEL of 30 mg/kg/day.

3.3.5.3 Chronic (> 12 months) toxicity

/

3.3.6 Mutagenicity / Genotoxicity

3.3.6.1 Mutagenicity / Genotoxicity *in vitro*

Bacterial Reverse Mutation Test

Guideline:	OECD TG 471
Species/Strain:	*Salmonella typhimurium* (TA1535, TA1537, TA98, TA100 and TA102)
Replicates:	Triplicates plates in two separate experiments
Test substance:	Hydroxypropyl p-phenylenediamine (base)
Batch:	R0025521A 001 L 002
Purity:	Relative purity 99.8%
Solvent:	Purified water
Positive controls:	Without S9 mix: 2-nitrofluorene (TA98), sodium azide (TA100, TA1535), 9-aminoacrridine (TA1537), mitomycin C (TA102) With S9 mix: benzo[a]pyrene (TA 100), 2-anthramine (TA1535, TA 1537, TA 98)
Concentrations:	Experiment 1 and 2: 0, 312.5, 625, 1250, 2500 and 5000 µg/plate without and with S9-mix.
Experiment 3:	0, 468.8, 625, 937.5, 1250, 2500 and 5000 µg/plate without and with S9-mix for the TA98 strain in presence of S9 mix

Treatment:	Direct plate incorporation incubated for 3 days protected from light without and with S9-mix. Experiments 2 and 3 with S9 mix were performed according to the pre-incubation method
GLP:	In compliance
Study period:	May – August 2015

The test item hydroxypropyl p-phenylenediamine was evaluated in two and three independent experiments in the absence and presence of metabolic activation, respectively (S9 mix prepared from the livers of rats given Aroclor 1254). The experiments were conducted according to the direct plating incorporation method, apart from the second and third tests with S9 mix which were performed according to the pre-incubation method. Since hydroxypropyl p-phenylenediamine was freely soluble at 50 mg/ml in water, concentrations used were as follows:

- 312.5, 625, 1250, 2500 and 5000 µg/plate in experiment 1 and 2, in the absence and presence of S9 mix
- 468.8, 625, 937.5, 1250, 2500 and 5000 µg/plate for the TA 98 strain in the third experiment, in the presence of S9 mix.

Known mutagens were used as positive controls, and cultures treated with purified water (solvent) were used as negative controls.

Results

All solvent and positive controls gave counts of revertants within expected ranges, and the experiments were therefore considered to be valid. Slight increases in the number of revertant colonies were observed in the TA 98 strain in the first experiment (up to 1.9-fold the vehicle control value). Since they did not reach the positive threshold of 2-fold the vehicle control value, were not dose-related and were not reproduced in the second experiment performed under the same experimental conditions, these increases were considered not to be biologically relevant.

A slight increase in the number of revertant colonies was observed at the dose-level of 625 µg/plate in the TA 98 strain in the second experiment (pre-incubation method). Since it did not reach the positive threshold of 2-fold the vehicle control value (1.9-fold), was not dose-related, was not observed using the direct plate incorporation method (first experiment) and was not reproduced in the third experiment (pre-incubation method) despite using a narrower range of dose-levels, this increase was considered not to be biologically relevant. No significant and reproducible increased number of revertants was observed with any other bacterial strain.

Conclusion

Under the conditions of this study, hydroxypropyl p-phenylenediamine was not mutagenic in *Salmonella typhimurium* strains TA1535, TA1537, TA98, TA100 and TA102 either in the presence or absence of metabolic activation.

Ref.: Sire, 2015

SCCS comment

The SCCS notes that in the experiment with hydroxypropyl p-phenylenediamine (base form) borderline positive results (1.9 fold) were obtained with strain TA 98 both without (in the first experiment) and with metabolic activation S9-mix (second experiment). In the second experiment negative control revertant values slightly exceeded the maximum of the historical negative control. Despite these limitations, the SCCS agrees that hydroxypropyl-p-phenylenediamine was not mutagenic in the bacterial reverse mutation test.

Bacterial Reverse Mutation Test

Guideline:	OECD TG 471
Species/Strain:	*Salmonella typhimurium* (TA1535, TA1537, TA98, TA100 and TA102)
Replicates:	Triplicates plates in two separate experiments; five plates for negative controls
Test substance:	Hydroxypropyl p-phenylenediamine HCl (salt)
Batch:	R0025521B 004 P 001
Purity:	Relative purity 98.6%
Solvent:	Purified water
Positive controls:	2-nitrofluorene (TA98), sodium azide (TA100, TA1535), 9-aminoacrridine (TA1537), mitomycin C (TA102), benzo[a]pyrene (TA98), 2-aminoanthracene (TA100, TA1535, TA1537, TA102)

Concentrations:	Experiment 1: 0, 5, 15.81, 50, 158.1, 500, 1581 and 5000 µg/plate without and with S9-mix. Experiment 2: 0, 20.48, 51.2, 128, 320, 800, 2000 and 5000 µg/plate without and with S9-mix for all strains except TA1537 (8.192, 20.48, 51.2, 128, 320, 800 and 2000 µg/plate)
Treatment:	Direct plate incorporation incubated for 3 days protected from light without and with S9-mix
GLP:	In compliance
Study period:	June 25 – November 13 2012

The test item hydroxypropyl p-phenylenediamine HCl was evaluated for the induction of gene mutations in *Salmonella typhimurium* (Ames test) in two independent experiments in the absence and presence of metabolic activation (S9 mix prepared from the livers of rats given Aroclor 1254). The experiments were both conducted according to the direct plating incorporation method. Since hydroxypropyl p-phenylenediamine HCl was freely soluble at 50 mg/mL in water, concentrations used were as follows:

- 5, 15.81, 50, 158.1, 500, 1581 and 5000 µg/plate in experiment 1, both in the absence and presence of S9 mix
- 20.48, 51.2, 128, 320, 800, 2000 and 5000 µg/plate in experiment 2, both in the absence and presence of S9 mix for all stains except strain TA1537 where cytotoxicity was noted at 5000 µg/plate, and therefore the chosen concentrations for this strain were: 8.192, 20.48, 51.2, 128, 320, 800 and 2000 µg/plate.
- Negative and positive controls were in accordance with the OECD guideline.

Results
No precipitation occurred up to the highest concentration investigated. All solvent and positive controls gave counts of revertants within expected ranges, and experiments were therefore considered to be valid. When compared to controls, a reproducible and significant increase in the number of revertants was observed for TA98 both in the absence and presence of S9 mix and to a lesser extent with strain TA1537 in the presence of S9. No significant and reproducible increased number of revertants was observed with any other bacterial strain.

Conclusion
Under the conditions of this study, hydroxypropyl p-phenylenediamine HCl was found mutagenic in *Salmonella typhimurium* strain TA98 in the presence or absence of metabolic activation and in strain TA1537 in the presence of metabolic activation.

Ref.: Hobson, 2012

***In vitro* micronucleus test in cultured human lymphocytes**

Guideline:	OECD TG 487
Cells:	Human lymphocytes
Replicates:	Duplicate cultures from two male volunteers in 2 independent experiments
Test substance:	Hydroxypropyl p-phenylenediamine (base)
Batch:	R0025521A 001 L 002
Purity:	Relative purity >98%
Solvent:	DMSO (experiment)
Positive controls:	Cyclophosphamide, Mitomycin C, Noscapine
Concentrations:	Experiment I: 3 h treatment + 21 recovery 0, 50, 100, 150, 200, 225, 250, 275, 300, 325, 350, 375, 400, 450 and 500 µg/mL without S9-mix 3 h treatment + 21 recovery 0, 25, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1250 with S9-mix 24 h treatment + 0 recovery 0, 10, 20, 30, 40 50, 55, 60, 65, 70, 75, 80, 90, 100 and 150 without S9-mix Experiment II: 3 h treatment + 21 recovery 0, 100, 150, 200, 225, 250, 275, 300, 325, 350, 375, 400, 450 and 500 µg/mL without S9-mix
Treatment:	Experiment I: 3 + 21 hour treatment both with and without S9-mix; Experiment II without S9-mix; test article added at 48 h following culture

	initiation (stimulation by PHA). Cultures were sampled 3 or 24 h after the beginning of the treatment (i.e. 72 h after culture initiation).
GLP:	In compliance
Study period:	14 October 2015 – April 25 2016

The test item hydroxypropyl p-phenylenediamine was evaluated in the absence and presence of metabolic activation (S9 mix prepared from the livers of Aroclor 1254-treated rats) in the first experiment. The highest concentration in each test condition was selected on the basis of the cytotoxicity and the following ranges of concentrations were selected for micronucleus analysis:

- 200, 275 and 325 µg/mL in 3h-treatment in the absence of S9
- 100, 400 and 600 µg/mL in 3h-treatment in the presence of S9
- 30, 40 and 55 µg/mL in 24h-treatment in the absence of S9

In the second experiment, only the condition 3h-treatment in the absence of S9 was tested to confirm the results obtained in the first experiment. The following concentrations (100 and 150 µg/mL) were selected for micronucleus analysis based on cytotoxicity.
Duplicate cultures were treated with each concentration of hydroxypropyl p-phenylenediamine or with known clastogens in the presence (cyclophosphamide, CPA) or absence of S9 (mitomycin C, MMC and Noscapine, NOS). Solvent-treated cultures (DMSO, four replicates) were used as negative controls.
Blood cultures were incubated in the presence of the mitogen phytohaemagglutinin (PHA) for 48 hours and then received a 24- or 3-hour treatment in the absence or presence of S9 mix, respectively. Cells were harvested 72 hours after the beginning of incubation. Cytochalasin B was added after the 3-hour treatments or before the 24-hour treatments.
Lymphocyte preparations were stained and examined microscopically for determining the replication index (RI) and the proportion of micronucleated binucleated (MNBN) cells when selected. Two thousand binucleate cells per concentration (one thousand from each replicate) were analysed blind.

Results
When compared to concurrent solvent controls, treatment of cultures with positive controls CPA, MMC and NOS resulted in consistent significant increases in MNBN frequencies, thus validating the sensitivity of the test system and procedure used.
Treatment of cultures at 3h-treatment in the absence of S9 with hydroxypropyl p-phenylenediamine resulted in frequencies of MNBN cells which were statistically significantly elevated when compared to concurrent solvent control cultures at the three tested concentrations. The MNBN cell frequency of a single treated culture at 275 µg/mL (inducing 23% cytotoxicity) and both treated cultures at 325 µg/mL (inducing 47% cytotoxicity) exceeded the 95th percentile of the normal ranges.
In a second confirmatory experiment, an increase in micronuclei following the 3-hour treatment without S-9 was observed with the two analysed concentrations (100 and 150 µg/mL, inducing 21% and 43% cytotoxicity respectively) where one of the two replicate cultures at each concentration demonstrated a MNBN cell frequency that exceeded the normal range (with the second cultures demonstrating MNBN cell values at the upper limit). Overall these data were considered additional evidence of a test article related effect. No such increases were observed following either the 3-hour treatment in the presence of S-9 or the 24-hour treatment in the absence of S-9 with concentrations analysed up to maximum inducing 53-55% cytotoxicity.

Conclusion
Under the conditions of the study, hydroxypropyl p-phenylenediamine induced micronuclei in cultured human peripheral blood lymphocytes after the 3-hour treatment in the absence of metabolic activation.

Ref.: Whitwell, 2016

***In vitro* micronucleus test in cultured human lymphocytes**

Guideline:	OECD TG 487
Cells:	Human lymphocytes from two female and follow up with two male volunteers
Replicates:	Duplicate cultures in 2 independent experiment
Test substance:	Hydroxypropyl p-phenylenediamine HCl (salt)
Batch:	R0025521B 004 P 001
Purity:	Relative purity 98.6%
Solvent:	Water (experiment I and II)

Positive controls:	Mitomycin C, Cyclophospamide, Vinblastine
Concentrations:	Experiment I: 3 h treatment + 21 h recovery 0, 10, 25, 50, 100, 150, 250, 500, 700, 800, 850, 900, 950, 1000, 1050 and 1100 µg/mL without S9-mix 3 h treatment + 21 h recovery 0, 50, 75, 150, 300, 450, 600, 800, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700 and 1800 µg/mL with S9-mix 24 h treatment + 0 h recovery 0, 10, 50, 70, 80, 90, 100, 110, 120, 130, 150, 175 200, 250, 300, 350 and 400 µg/mL without S9-mix Follow up experiment: 3 h treatment + 21 h recovery 0, 10, 50, 100, 250, 500, 550, 600, 650, without S9-mix 700, 760, 800, 850, 900, 950, 1000, 1050, 1100 and 1200 µg/mL
Treatment:	Experiment I: 3 h + 21 h treatment both with and without S9-mix; test article added at 48 h following culture initiation (stimulation by PHA). Cultures were sampled 3 or 24 h after the beginning of the treatment (i.e. 72 h after culture initiation). Follow up experiment: 3 + 21 hour treatment without S9-mix;
GLP:	in compliance
Study period:	6 July – 3 June 2014

Hydroxypropyl p-phenylenediamine HCl has been investigated for the induction of micronuclei in cultured human lymphocytes. The study was conducted on cultured human peripheral blood lymphocytes from two female volunteers. A follow-up study was conducted on cultured human peripheral blood lymphocytes from two male volunteers.

The test item hydroxypropyl p-phenylenediamine HCl was evaluated in the absence and presence of metabolic activation (S9 mix prepared from the livers of Aroclor 1254-treated rats). The highest concentration in each test condition was selected on the basis of the cytotoxicity and the following ranges of concentrations were selected for micronucleus analysis:

- 25, 250, 700, 850 and 1050 µg/mL in 3h-treatment in the absence of S9
- 450, 1300 and 1600 µg/mL in 3h-treatment in the presence of S9
- 10, 80 and 110 µg/mL in 24h-treatment in the absence of S9

In a follow-up study, only the condition 3h-treatment in the absence of S9 was tested due to questionable results in a single experiment. The following ranges of concentrations were selected for micronucleus analysis based on cytotoxicity:

- 250, 650, 850 and 900 µg/mL in 3h-treatment in the absence of S9

Duplicate cultures were treated with each concentration of hydroxypropyl p-phenylenediamine HCl or with known clastogens in the presence (cyclophosphamide, CPA) or absence of S9 (mitomycin C, MMC and vinblastine, VIN). Solvent-treated cultures (purified water, four replicates) were used as negative controls.
Blood cultures were incubated in the presence of the mitogen phytohaemagglutinin (PHA) for 48 hours and then received a 24- or 3-hour treatment in the absence or presence of S9 mix, respectively. Cells were harvested 72 hours after the beginning of incubation. Cytochalasin B was added after the 3-hour treatments or before the 24-hour treatments.
Lymphocyte preparations were stained and examined microscopically for determining the replication index (RI) and the proportion of micronucleated binucleated (MNBN) cells when selected. Two thousand binucleate cells per concentration (one thousand from each replicate) were analysed blind.

Results
The solubility limit in culture medium was considered close to 5094 µg/mL, as indicated by a lack of visible precipitation upon test article addition, but which was observed following a 20 hour incubation period. Measurements on post-treatment media in the absence or presence of S9-mix indicated that test compound had effect on osmolarity or pH in higher concentrations as compared to concurrent vehicle controls. As concentrations analysed for micronuclei from the main experiment did not exceed 1600 µg/mL, these observations were not considered to have had any impact on the interpretation of the results data.
When compared to concurrent solvent controls, treatment of cultures with positive controls CPA, MMC and VIN resulted in consistent significant increases in MNBN frequencies, thus validating the sensitivity of the test system and procedure used.

Treatment of cultures at 3h-treatment in the absence of S9 with hydroxypropyl p-phenylenediamine HCl resulted in frequencies of MNBN cells which were statistically significantly elevated when compared to concurrent solvent control cultures at the highest two concentrations analysed (850 and 1050 µg/mL exhibiting 48% and 60% cytotoxicity, respectively).
In a follow-up study, the previous condition was repeated. Treatment of cells with hydroxypropyl p-phenylenediamine HCl in the absence of S9 resulted in small but statistically significant increases of MNBN at the two intermediate concentrations (650 and 850 µg/mL inducing 30% and 53% cytotoxicity, respectively). These statistical increases were noted to be set against a low concurrent vehicle control response. Moreover, they were small and fell within normal ranges. As such, these data are considered of questionable biological importance.
Overall, given the poor reproducibility of response between the experiments, the ability of hydroxypropyl p-phenylenediamine HCl to induce biologically relevant increases in MNBN cells is considered questionable.

No statistically significant increases were observed following continuous 24-h without S9 treatment with hydroxypropyl p-phenylenediamine HCl. Slight increase in MNBN cell frequency was observed in the presence of S9 at the intermediate concentration (1300 µg/mL) in only one replicate. No increase in MNBN was observed at the lowest and highest concentrations (450 and 1600 µg/mL, respectively). Therefore, given the minimal magnitude, the absence of a dose-response and the isolated increase, the latter was considered to be of no biological significance.
In experiment 2 R0025521B induced, small statistically significant increases in micronuclei at two intermediate concentrations analysed following a 3 hour treatment with 21 hour recovery in the absence of a rat liver metabolic activation system (S-9).
As these increases were small and set against a low concurrent vehicle control response with individual micronucleated cell values for all R0025521B treated cultures falling within historical vehicle control ranges, these statistical increases were considered of questionable biological relevance.

Conclusion
Under the conditions of the study, hydroxypropyl p-phenylenediamine HCl produced a poorly reproducible increase in the micronuclei in cultured human peripheral blood lymphocytes in the 3-h treatment in the absence of metabolic activation, which is considered of questionable biological relevance.

Ref.: Whitwell, 2014

SCCS comment
The SCCS considers results from the micronucleus test on hydroxypropyl p-phenylenediamine 2HCl as positive.
In experiment 1, hydroxypropyl p-phenylenediamine 2HCl induced a significantly higher frequency of micronuclei in cultured human peripheral blood lymphocytes following 3+21 hour treatment in the absence of a rat liver metabolic activation system (S9-mix). A concentration-related increase in micronucleus frequency was also noted following continuous 24+0 hour –S9-mix treatment.
Micronucleus frequency in the vehicle controls treated for 24 h exceeded the historical control. Precipitation was observed at the end of treatment with and without S9-mix and also at the harvest time after 24 h exposure without S9-mix.
In the follow up experiment hydroxypropyl p-phenylenediamine 2HCl induced small but statistically significant increases in micronuclei at two concentrations analysed after a 3-hour treatment with a 21-hour recovery in the absence of a rat liver metabolic activation system (S9-mix).

Mammalian cell gene mutation test (*Hprt* locus) in mouse lymphoma cells

Guideline:	OECD TG 476
Cells:	Mouse lymphoma cell line L5178Y [*Hprt* locus for 6-thioguanine (6-TG) resistance]
Replicates:	Duplicate cultures in two independent experiments
Test substance:	Hydroxypropyl p-phenylenediamine HCl (salt)
Batch:	R0025521B 004 P 001
Purity:	Relative purity 98.6%
Solvent:	Purified water diluted 10-fold in treatment medium
Positive controls:	4-nitroquinoline 1-oxide (NQO), benzo[a]pyrene (BP)

Concentrations:	Experiment 1: 0, 25, 50 and 75 µg/mL without S9-mix; 0, 100, 200, 250, 300, 350, 400, 425 and 450 µg/mL with S9-mix Experiment 2 0, 30, 40, 50, 60, 70, 80, 90, 100 and 125 µg/mL without S9-mix; 0, 100, 200, 300, 350, 375, 400, 450 and 500 µg/mL with S9-mix
Treatment:	Experiment 1 and 2: 3 h treatment both without and with S9-mix; expression period 7 days
GLP:	In compliance
Study period:	July 9 2012 – February 8 2013

The test item hydroxypropyl p-phenylenediamine HCl was evaluated for gene mutations at the *Hprt* locus of L5178Y cells in two independent experiments using duplicate cultures each (single cultures for positive controls). Cytotoxicity was evaluated by range-finder experiment followed by two independent experiments. Both experiments used a pulse (3-hour) treatment and were conducted in the absence and presence of metabolic activation (S9 mix prepared from the liver of rats given Aroclor 1254). The ingredient hydroxypropyl p-phenylenediamine HCl was tested in both experiments at a broad range of concentrations. The concentrations selected on the basis of cytotoxicity criteria for final test statistics were as follows:

Experiment 1
- In the absence of S9 mix: 25, 50 and 75 µg/mL (13% relative survival at 75 µg/mL)
- In the presence of S9 mix: 100, 200, 250, 300, 350, 400, 425 and 450 µg/mL (11% relative survival at 450 µg/mL)
-

Experiment 2
- In the absence of S9 mix: 30, 40, 50, 60, 70, 80, 90, 100 and 125 µg/mL (13% relative survival at 125 µg/mL)
- In the presence of S9 mix: 100, 200, 300, 350, 375, 400, 450 and 500 µg/mL (13% relative survival at 500 µg/mL)

Known mutagens in the presence (BP) or absence of S9 mix (NQO) were tested at two different concentrations and served as positive controls. Negative controls consisted of cultures treated with the solvent alone (purified water). Cells were suspended in culture medium and exposed to various concentrations of the test item, to solvent or positive controls. After the treatment period (3 hours), the cells were resuspended in culture medium. They were transferred to flasks for growth through the expression period (7 days) or were diluted to be plated for survival (7 to 8 days). At the end of the expression period, acceptable cultures were then plated for viability (2 plates per culture, 7 days) or 6-TG resistance (4 plates per culture, 11 days).

Results
Mutant frequencies in solvent negative controls fell within normal ranges, and treatment with positive controls NQO and BP yielded distinct increases in mutant frequency. Accordingly, the study was considered to be valid.
When tested up to the limit of cytotoxicity, there were no statistically significant increases in mutant frequency and no significant linear trends following treatment with hydroxypropyl p-phenylenediamine HCl at any concentration tested in the absence and presence of metabolic activation S9-mix.

Conclusion
Under the conditions of this study, hydroxypropyl p-phenylenediamine HCl was considered not to be mutagenic in the mouse lymphoma assay (*Hprt* locus), either in the absence or presence of metabolic activation.

Ref.: Lloyd, 2013

SCCS comment
The SCCS does not agree that hydroxypropyl p-phenylenediamine 2HCl is not mutagenic in this assay.
In experiment 1, only 3 concentrations were used for mutagenicity assessment in the absence of S9-mix. According to the study director, the fourth concentration, which induced higher mutant frequency, was excluded due to high cytotoxicity. However, the cytotoxicity was within the recommended range (survival more than 10% versus 100% of control). These test results should

therefore be included in the assessment. In addition, a statistically significant linear trend in mutant frequency was observed in the presence of S9-mix in Experiment 2.
The SCCS questions the validity of this assay, due to a high variability between the experiments. More specifically, SCCS noticed that there was a high variability in the mutant frequency in negative controls between the two experiments. Especially in the presence of S9-mix the difference is almost an order of magnitude, i.e. 8.15 in experiment 1 (only one replicate) vs. 0.87 in Experiment 2. Similarly, the mutant frequency in cells treated with 50 µg/mL hydroxypropyl p-phenylenediamine 2HCl without S9-mix differed considerably between the experiments (9.17 in Exp1 vs. 0.55 in Exp2). Negative controls (both with and without S9-mix) in experiment 1 were higher than the reported historical controls. Additionally, the effect of positive control NQO was highly variable between experiments: with mutant frequency 70 and 221 in Experiment 1 and 7.8 and 12.7 in Experiment 2.

Due to the high variability between the experiments, the SCCS concluded that the results of this test have limited value and a genotoxic potential for hydroxypropyl p-phenylenediamine 2HCl cannot be excluded. In May 2018, the SCCS sent a request to the Applicant for an additional mammalian cell gene mutation test that would fulfil the acceptance criteria. Results of this new test were submitted to the SCCS in March 2019 and are described below. The Applicant also provided complementary evidence from *in silico* models and a read-across approach conducted with structural analogues. This approach is described in this Opinion as well.

Mammalian cell gene mutation test (*Hprt* locus) in mouse lymphoma cells

Guideline:	OECD TG 476
Cells:	Mouse lymphoma cell line L5178Y [*Hprt* locus for 6-thioguanine (6-TG) resistance]
Replicates:	Duplicate cultures in two independent experiments
Test substance:	Hydroxypropyl p-phenylenediamine HCl (salt)
Batch:	R0025521B 004 P 001
Purity:	Relative purity 98.6%
Solvent:	Purified water diluted 10-fold in treatment medium
Positive controls:	4-nitroquinoline 1-oxide (NQO), benzo[a]pyrene (BP)
Concentrations:	Experiment 1: 0, 20, 40, 80, 100, 120, 150 and 200 µg/mL without S9-mix; 0, 100, 200, 250, 300, 350 and 400 µg/mL with S9-mix Experiment 2: 0, 20, 40 and 60 µg/mL without S9-mix (6% RS µg/mL); repeated due to high cytotoxicity: 0, 15, 30, 40, 50, 60, 80, 100, 125 and 150 µg/mL without S9-mix
Treatment:	Experiment 1: 3 h treatment both without and with S9-mix; Experiment 2: 3 h treatment without S9-mix; Expression period 7 days
GLP:	In compliance
Study period:	August 8, 2018 – November 21, 2018

R0025521B was assayed for the ability to induce mutation at the hypoxanthineguanine phosphoribosyl transferase (*Hprt*) locus (6-thioguanine [6TG] resistance) in mouse lymphoma cells using a fluctuation protocol. The study consisted of a cytotoxicity range-finder experiment followed by two mutation experiments.
Experiment 1 was conducted in the absence and presence of metabolic activation by an Aroclor 1254-induced rat liver post-mitochondrial fraction (S-9).
Experiment 2 was conducted in the absence of S-9, to clarify the results observed under this treatment condition in Experiment 1. The test article was formulated in purified water. A 3 hour treatment incubation period was used for each experiment.

Results
In the cytotoxicity Range-Finder Experiment, six concentrations were tested in the absence and presence of S-9, ranging from 62.5 to 2000 µg/mL. The highest concentrations to give >10% relative survival (RS) were 125 µg/mL in the absence of S9-mix and 250 µg/mL in the presence of S9-mix, which gave 11% and 84% RS, respectively. Mutant frequencies in solvent negative controls fell within normal ranges, and treatment with positive controls NQO and BP yielded distinct increases in mutant frequency. Accordingly, the study was considered to be valid.

In Experiment 1 in the presence of S9-mix, no statistically significant increases in MF were observed at any R0025521B concentration analysed. A statistically significant linear trend ($p \leq 0.05$) was observed but as there were no significant increases in MF at any concentration analysed, the observations were considered not biologically relevant. In Experiment 1 in the absence of S9-mix, a statistically significant increase in MF was observed at the highest R0025521B concentration analysed (200 µg/mL, giving 11% RS) and there was a statistically significant linear trend ($p \leq 0.05$). The mean MF value at 200 µg/mL was 6.64 mutants per 10^6 viable cells (individual values were 7.33 and 5.83 mutants per 10^6 viable cells).
The historical vehicle control range (based on the last 20 experiments performed in this laboratory, prior to Experiment 1) is 0.73 to 6.65 mutants per 10^6 viable cells, therefore the mean MF value at 200 µg/mL was marginally within the historical vehicle control range and only one culture at this concentration marginally exceeded the range. The response did not fulfill all of the evaluation criteria for a positive result, although there was evidence of an increase in MF, albeit at this concentration alone which was close to the upper limit of toxicity (11% RS) for this type of study.
In Experiment 2 in the absence of S9-mix, a statistically significant increase in mutant frequency (MF) was observed at one intermediate R0025521B concentration analysed and there was no statistically significant linear trend. The mean MF value at 50 µg/mL was 5.70 mutants per 10^6 viable cells, which marginally exceeded the historical vehicle control range (based on the last 20 experiments performed in this laboratory, prior to Experiment 2 repeat) of 0.49 to 5.47 mutants per 10^6 viable cells. However, as in Experiment 1, the response did not fulfill all of the evaluation criteria for a positive result. Based on the data over the two experiments in the absence of S-9-mix, R0025521B showed some evidence of inducing mutations in this test system in both experiments. However, the increases in mutant frequency were small in magnitude and in one experiment the response was not concentration-related (as evidenced by a non-significant linear trend). The responses in both experiments did not fulfill all of the evaluation criteria for a positive result. As the data were poorly reproducible, they may therefore be considered of no toxicological concern.

Conclusion
It is concluded that R0025521B did not induce biologically relevant increases in mutant frequency at the *Hprt* locus of L5178Y mouse lymphoma cells in two independent experiments when tested up to toxic concentrations in the absence and presence of a rat liver metabolic activation system (S-9) under the experimental conditions described.

Ref: Lloyd, 2019

SCCS comment
The SCCS does not agree that hydroxypropyl p-phenylenediamine 2HCl is not mutagenic in this additional mammalian gene mutation assay. In Experiment 1 in the absence of S9-mix, a statistically significant increase in mutant frequency was observed at the highest concentration analysed and there was a statistically significant linear trend. Experiment 2, in the absence of S9-mix, was considered as invalid, because not enough concentrations could be analysed. Therefore, the second experiment was repeated. In Experiment 2 repeat, in the absence of S9-mix, a statistically significant increase in mutant frequency was also observed at one intermediate concentration, but without a positive linear trend.
The reliability of this additional mammalian gene mutation test is questionable because variability between the experiments was high. Furthermore, the data on the historical positive control show a high variability. More specifically, the following issues were observed by the SCCS:

- The different negative historical control ranges reported in this study, based on the mutation frequency observed during the last 20 experiments, were different for Experiment 1 and the repeated Experiment 2. The calculations of the ranges and why these are not the same are not clear and need explanation.
- A high variation in mutant frequency of the positive control NQO was noticed between the experiments. NQO induced MF of 65.7-73.8/10^6 cells in Experiment 1, 5.4-12.14/10^6 cells in Experiment 2 and of 6-12.72/10^6 cells in the repeated Experiment 2. Similarly to the previous mammalian gene mutation test (Lloyd 2013), the mutation frequency for the positive control differed an order of magnitude between the two experiments (in the Experiment 2 repeat, it was almost at the vehicle control level).
- In line with this, a high variation in MF for NQO historical positive control was observed, ranging from 0.33 to 82.62/10^6 cells.

Taken together, the newly submitted test did not meet the acceptance criteria as described in the study report §4.4.1. p.2 and OECD TG 476. The observed variability in results with the NQO positive control indicates that the cell system generates unreliable responses. Therefore, the SCCS considers this test as not acceptable and the results cannot be used to exclude a mutagenic potential for hydroxypropyl p-phenylenediamine 2HCl.

3.3.6.2 Mutagenicity / Genotoxicity *in vivo*

Integrated bone marrow micronucleus test in a 14-day oral toxicity study in rats

Guideline:	/
Species/strain:	Wistar rats
Group size:	5/sex/group
Test substance:	Hydroxypropyl p-phenylenediamine HCl (salt)
Batch:	R0025521B 004 P 001
Purity:	98.6%
Vehicle:	Distilled water
Dose levels:	0, 100, 300 or 1000 mg/kg
Dose volume:	10 mL/kg
Route:	Oral
Administration:	Gavage
GLP:	In compliance
Study period:	May 2012 – June 2012

In the main study, rats (5/sex/group) received a 14-day repeated oral (gavage) dose of hydroxypropyl p-phenylenediamine HCl at 0, 100, 300 or 1000 mg/kg in distilled water at 10 mL/kg. Animals from hydroxypropyl p-phenylenediamine HCl or vehicle control groups were killed 24 hours after the last administration. For each animal, smears were prepared from femur bone marrow and were scored blind for the incidence of micronucleated polychromatic erythrocytes (MN-PCE, 2000 PCE counted); the polychromatic/normochromatic erythrocyte ratio (PCE/NCE) was also determined.

Results
Mortality was observed in the high and mid dose groups (10/10 and 1/10, respectively). Rats in the mid dose group (300 mg/kg) showed significant reductions in body weight gain compared to controls, from day 4 onwards in males and from day 8 onwards in females. Yellow to yellowish brown colored urine was also observed, confirming the systemic exposure to hydroxypropyl p-phenylenediamine HCl. Thus, the dose level of 300 mg/kg can be considered a maximum tolerated dose. The incidence of MN-PCE was similar for hydroxypropyl p-phenylenediamine HCl-treated and control animals at both dose levels of 100 and 300 mg/kg. There was no evidence of bone marrow toxicity in animals given hydroxypropyl p-phenylenediamine HCl as the PCE/NCE ratio was similar among groups.

Conclusion
Under the conditions of this study, hydroxypropyl p-phenylenediamine HCl did not induce cytogenetic damage leading to micronucleus formation in the bone marrow of rats treated orally in a 14-day repeated toxicity study up to the maximum tolerated dose of 300 mg/kg.

Ref.: Mehta, 2013

Overall conclusion on mutagenicity by the Applicant
Overall, hydroxypropyl p-phenylenediamine and its dihydrochloride salt were considered to have no genotoxic potential *in vivo* after testing all required endpoints of genotoxicity, i.e. a) gene mutations (bacteria, mouse lymphoma cells), b) clastogenicity and c) aneugenicity (*in vitro* micronucleus test in cultured human lymphocytes, rat bone marrow micronucleus test integrated into a 14-day repeated oral toxicity study).

It has to be noted that hydroxypropyl p-phenylenediamine, a p-phenylenediamine derivative, is structurally very close to other well-known dyes e.g. toluene-2,5-diamine (A5), hydroxyethyl-p-phenylenediamine sulphate (A80) and 2-Methoxy-methyl-p-phenylenediamine (A160). In particular, A80 is structurally very close as A165 has one extra Methyl on its side chain compared to A80. This family of hair dyes was largely tested in mutagenicity and genotoxicity assays (Ames, MLA *Tk* and

Hprt locus, micronucleus, chromosome aberration or UDS tests) and the positive *in vitro* results were not confirmed *in vivo*.

Similarly, on the basis of the weight of evidence, hydroxypropyl p-phenylenediamine and its dihydrochloride salt are expected to be devoid of genotoxic potential *in vivo*.

Supporting evidence from *in silico* models and read-across approach on mutagenicity
Upon a request by the SCCS, that the selection of structural analogues to A165 should be more formally documented and reported, the Applicant provided supporting evidence from read-across and QSARs to exclude mutagenicity of hydroxypropyl p-phenylenediamine.
The Applicant undertook a formal search to retrieve structural analogues of hydroxypropyl p-phenylenediamine using the OECD QSAR Toolbox (v4.2), as recommended by the European Chemical Agency and the recent 10th SCCS Notes of Guidance (SCCS, 2018c). The outcome of this search is outlined below, with additional, complementary Tables included in Appendix 1.

The databases used were:
- All of the databases listed in the OECD Toolbox under "human health" criterion
- An inventory containing 158 oxidative and non-oxidative hair dye ingredients, corresponding to all of the hair dye ingredients evaluated by SCCS/SCCP/SCCNFP. This inventory was implemented in the Toolbox by the applicant.

Analogues were searched on the basis of:
1- Organic Functional Groups (OFGs), as defined in the 4 grouping methods of OECD QSAR Toolbox v4.2 (OFG, OFG Nested, OFG US EPA, OFG Norbert Haider). In order to avoid retrieval of molecules with functional groups non present in A165, the search was done by selecting the 'strict' criterion, restricting the search to analogues having the relevant functional groups present in the target chemical molecule A165.

<u>*Outcome of the search for analogues*</u>: Only one analogue (Cosmetics EU N°A080, CAS RN 93841-25-9 and 93841-24-8) was found with the selection criteria mentioned above

2- Structural similarity: the selection criteria used considered that the analogue had to be more than 70% similar to the parent compound, which is often taken as a cut-off for read-across (Price and Chaudhry, 2014; Hartung, 2016). Structural similarity of analogues was assessed by using the similarity modules in the QSAR Toolbox. The parent compound A165 was submitted to a search for >70% similarity based on the Dice or Tanimoto algorithms, using atom centered as the basis for comparison.

<u>*Outcome of the search for analogues:*</u>
a. When Tanimoto algorithm (atom centered) was used, only A080 (described previously) was found
b. With the default criteria in the Toolbox (Dice atom centered), 3 analogues: A080, A042 HCl (2,4-Diaminophenoxyethanol hydrochloride, CAS RN 66422-95-5), and A042 base (2,4-Diaminophenoxyethanol, CAS RN 70643-19-5) were identified

Overall, two analogues were identified by following the above selection method:
- A080 (Hydroxyethyl-p-phenylenediamine, CAS RN 93841-24-8; Hydroxyethyl-p-phenylenediamine sulphate CAS RN 93841-25-9) was selected when both criteria of category definition (*i.e.* OFGs and structural similarity) were used. A080 is a hair dye ingredient authorized in EU (Entry N°206 in Annex III of the EU Cosmetic Products Regulation), which was concluded to be safe by the SCCS (SCCS, 2010a).
- A042 (2,4-Diaminophenoxyethanol base, CAS RN 70643-19-5; 2,4-Diaminophenoxyethanol hydrochloride, CAS RN 66422-95-5) was retrieved based on 70-80% structural similarity to A165. A042 is a hair dye ingredient authorized in EU (Entry N°242 in Annex III of the EU Cosmetic Products Regulation), which was concluded to be safe by the SCCS (SCCS, 2010b).

It must be noted that the formal search for analogues of A165 reported above retrieved fewer chemicals than the more informal search initially conducted by the Applicant, which retrieved three additional hair dye ingredient molecules (A007, A005, and A160). On the other hand, the present search retrieved one additional analogue (A042) that was not identified through the previous more

informal search, which is related to the less obvious chemical analogy between A042 and the target chemical A165. However, given that the three previously identified molecules have an overall genetic toxicity profile similar to A165 and to the two analogues identified here (A042 & A080), this slight discrepancy was considered not to change the overall conclusions of the present read-across approach.

The three chemicals A165, A080 and A042 were compared via the profiling of their OFG (Appendix 1, Table 1), their physico-chemical properties (Appendix 1, Table 2), the mutagenicity and genotoxicity alerts generated with the Times model v2.27.19 (Appendix 1, Table 3) and DNA binding, mutagenicity, genotoxicity alerts and oncologic classification generated using OECD QSAR Toolbox v4.2 (Appendix 1, Table4).

- The three chemicals considered are phenylenediamine-derivatives. The OFG in A165 and A080 are strictly identical using the 4 models in the OECD QSAR Toolbox. A042 has a phenylenediamine in meta-position instead of para when compared to the two other chemicals and showed additional OFG such as ether or one aromatic oxygen (Appendix 1, Table 2).
- The three chemicals have Molecular Weights in a narrow range (152-168 g/mol) and show comparable hydrophobicity characteristics, with calculated clogP values in the range -1.12 up to -0.74. All chemicals showed two ionizable amine function in the same range of pH value. The three chemicals showed similar profiles regarding volatility and water solubility over a wide range of pH values. The apparent changes in A165 water solubility classification with pH are due to the cut-off value of 33000 mg/L used to distinguish soluble and sparingly soluble chemicals, and are of no significance as confirmed when comparing the calculated values (Appendix 1, Table 3).
- The three hair dye ingredients A165, A080 and A042 have very similar genotoxicity profiles. All exhibit the same profile for reactivity towards DNA and in various models for gene mutation or carcinogenic classification (Appendix 1, Table 4). The only difference observed is that A042 was additionally predicted as non-genotoxic *in vivo* in the micronucleus Times model. This negative prediction was attributed to a detoxifying pathway related to a specific OFG of the chemical ("Single-Ring Aminophenol and Alkoxyaniline Derivatives", see Appendix 1, Table 5).

Based on the overall similarity of OFG, CPC and genotoxicity profiles of the chemicals A080 and A042 *versus* the target chemical A165, A080 and A042 were deemed to be suitable analogues to predict the mutagenic/genotoxic potential of A165.
Table 1 below summarizes all available *in vitro* and *in vivo* genetic toxicity data available for the target chemical A165 and its two analogues A042 & 1080. The hair dye ingredient A165 was positive in the *in vitro* micronucleus assay and clearly negative in the *in vivo* micronucleus assay. These findings are consistent with the *in vitro* and/or *in vivo* data available for the two analogues A080 and A042. Regarding the potential of these chemicals to produce gene (point) mutations, A080 was negative in both bacteria (Ames test) and mammalian cells (MLA, *Hprt* locus). The hair dye ingredient A042 was positive in the Ames test, but these positive *in vitro* findings were considered not to be biologically relevant on the basis of the absence of genotoxic and/or carcinogenic potential in mammalian cells (MLA, *Hprt* locus) and in two *in vivo* assays. Accordingly, on the basis of the results obtained with A165 in *in vitro* mutagenic *assays*, and when compared to those obtained with A080 and A042, A165 is expected to be devoid of gene mutagenic potential in mammalian cells under *in vivo* conditions.

In conclusion, the absence of gene mutation potential in mammalian cells and *in vivo* of both structural analogues A080 and A042 support the applicant's view that A165 has no gene mutation potential in mammalian cells, thus no such potential *in vivo*. Overall, the genotoxic profiling of A165, A080 and A042 reveals some genotoxicity *in vitro* alerts. However, these *in vitro* alerts do not result in a genetic toxicity potential under *in vivo* conditions, *i.e*. all *in vivo* assays are negative.

SCCS comment
The SCCS has noted that the Applicant used a systematic read-across and an *in silico* approach and has no comments on the procedures followed.
The QSAR Toolbox gave structural alerts for *in vitro* mutagenicity for both A165 and A080. Furthermore, hydroxypropyl p-phenylenediamine was positive according to the *in vitro* and *in vivo* predictions from the micronucleus Times model. These predictions are in line with the results obtained in the *in vitro* assays performed. Although in theory the positive results obtained from the

read-across *in silico* modelling or the *in vitro* micronucleus test could be overruled by the negative *in vivo* micronucleus test, the SCCS cannot exclude a genotoxic potential of hydroxypropyl p-phenylenediamine in the absence of a valid mammalian gene mutation test.

SCCS overall comment on mutagenicity
Mutagenicity of hydroxypropyl p-phenylenediamine (base) and its dihydrochloride salt was investigated in *in vitro* genotoxicity tests for the 3 endpoints of genotoxicity: gene mutations, structural and numerical chromosome aberrations. Hydroxypropyl p-phenylenediamine (base) was evaluated in the bacterial reverse mutation test and the *in vitro* micronucleus test in cultured human lymphocytes. Hydroxypropyl p-phenylenediamine 2HCl was evaluated in the bacterial reverse mutation test, two mammalian cell gene mutation tests in mouse lymphoma cells (*Hprt* locus) and a micronucleus assay in cultured human lymphocytes. Hydroxypropyl p-phenylenediamine 2HCl was tested in the mammalian *in vivo* micronucleus test integrated into a 14-day repeated oral toxicity study as well. The base form was not tested *in vivo*. Table 11 summarises the results of the genotoxicity tests.

Hydroxypropyl p-phenylenediamine (base) was negative in the bacterial reverse mutation test, but positive in the *in vitro* micronucleus assay and is considered to be an *in vitro* mutagen. Based on the data provided, a mutagenic potential cannot be excluded for the base form.

Hydroxypropyl p-phenylenediamine 2HCl was positive in the bacterial reverse mutation test and the *in vitro* micronucleus test. The *in vivo* micronucleus assay was negative and provides sufficient evidence that hydroxypropyl p-phenylenediamine 2HCl does not induce chromosomal damage and clastogenicity and is not genotoxic *in vivo*.

The *in vitro* mammalian gene mutation test was considered to be inconclusive by the SCCS due to several experimental limitations, including high variability in mutant frequency of positive control NQO between the experiments, a high variation in MF for NQO historical positive control and different negative historical control ranges reported for Experiment 1 and 2. The additional mammalian gene mutation test submitted by the Applicant was again unacceptable. Therefore, neither of these mammalian gene mutation studies can be used to exclude a gene mutation potential. In the absence of a valid mammalian gene mutation test, the SCCS cannot exclude a genotoxic potential of hydroxypropyl p-phenylenediamine.

Table 11: Summary of the results of the *in vitro* and *in vivo* genotoxicity studies

Test method	Hydroxypropyl p-phenylenediamine (base)	Hydroxypropyl p-phenylenediamine 2HCl
In vitro		
Gene mutation in bacteria (Ames test)	Negative	Positive
Mammalian cell gene mutation test	Not tested	Inconclusive
Mammalian cell gene mutation test (2019)	Not tested	Inconclusive
Micronucleus test	Positive	Positive
In vivo		
Mammalian micronucleus test	Not tested	Negative

3.3.7 Carcinogenicity

Cell Transformation Assay in Syrian Hamster Embryo Cells (SHE Assay)

Guideline:	/
Cells:	Viable and lethally irradiated Syrian Hamster Embryo cells (SHE)
Replicates:	
Test substance:	Hydroxypropyl p-phenylenediamine HCl
Batch:	R0025521B 004 P 001
Purity:	relative purity 99.3%
Solvent:	Complete cell culture medium
Positive controls:	Benzo[a]pyrene (B[a]P)
Concentrations:	0, 12, 14, 16, 18, 20, 22 and 24 µg/mL
Study period:	18 September – 25 November 2013

The ingredient hydroxypropyl p-phenylenediamine HCl was tested in a single experiment at a broad range of concentrations. The concentrations selected on the basis of cytotoxicity criteria were as follows: 12, 14, 16, 18, 20, 22 and 24 µg/mL.
Hydroxypropyl p-phenylenediamine HCl and the positive control were subsequently added to complete medium containing the target cells seeded onto feeder cells (x-ray irradiated SHE cells). The cultures were incubated for 7 days ± 2 hours. After the incubation period, the target cells were fixed with methanol and then stained with Giemsa. For the transformation experiment, 40 dishes with an expected colony count of 25-45 colonies were seeded per dose group. For the range finding experiment 10 dishes were seeded per dose group. SHE cells were examined microscopically for determining the plating efficiency (PE) and the morphological transformation (MT). At least one thousand (1000) colonies were evaluated for MT for each concentration.
B[a]P was used as positive control, and cultures with complete medium were used as negative controls.

Results
MT in medium negative control fell within normal ranges, and treatment with positive control B[a]P yielded statistically significant increase in morphologically transformed colonies. Accordingly, the study was considered to be valid. No relevant increase in the osmolarity or pH value (6.7) was observed. Cytotoxicity, defined as reduced relative plating efficiency of ≤ 50% compared to the control, was observed at the three highest evaluated dose groups (*i.e*. 20, 22 and 24 µg/mL). No statistically significant increases in the frequency of morphologically transformed colonies were observed up to the highest tested concentration.

Conclusion
Under the conditions of this study, hydroxypropyl p-phenylenediamine HCl did not induce morphological transformation of SHE cells colonies after 7 days of exposure.

Ref.: Bohnenberger, 2015

SCCS comment
SCCS has noted the results of the CTA test. According to the SCCS Notes of Guidance, the carcinogenic potential of a substance cannot be derived from a stand-alone CTA test. Therefore, no conclusions can be made on the carcinogenic potential of hydroxypropyl p-phenylenediamine 2HCl.

3.3.8 Reproductive toxicity

3.3.8.1 Two generation reproduction toxicity

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3.3.8.2 Other data on fertility and reproduction toxicity

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3.3.8.3 Developmental Toxicity

Oral Embryo-foetal Development Toxicity Study

Guideline:	OECD TG 414
GLP:	In compliance
Species/strain:	Rat/RccHanTM: WIST; 12 weeks old
Group size:	25 mated females per dose group
Test substance:	Hydroxypropyl p-phenylenediamine HCl
Batch:	R0025521B 004 P 001
Vehicle:	Reverse osmosis water
Dose level:	10, 30, 100 mg/kg bwt
Dose volume:	10 ml/kg bwt
Administration:	gavage
Study period:	21 December 2012 – 13 March 2014 (the experimental phase was from December 2012 to January 2013. The corresponding study report was completed in March, 2014)

Test Material
Hydroxypropyl p-phenylenediamine HCl batch 004 P 001 (relative purity 98.6%) was used in this study.

Test System
One hundred mated female Wistar rats were used.

Test Procedure
The potential effects of hydroxypropyl p-phenylenediamine HCl on pregnant rats and embryo-foetal development were evaluated through daily oral gavage in which mated Wistar female rats (25/group) were dosed at 0, 10, 30 or 100 mg/kg/day during the sensitive period of organogenesis from gestation day 5 to day 19 [the day of mating was designated as Gestation Day 0 (GD 0)]. The test item was dissolved in water and given at 10 mL/kg. These dose levels were selected on the basis of the results of a preliminary study 14-day study performed at 100, 300 and 1000 mg/kg/day where mortality was observed at the high and mid doses. No mortality, clinical signs, or relevant changes in body weight, body weight gain or food consumption were observed in the low dose group (100 mg/kg/day). Maternal evaluations and measurements included daily clinical signs and body weight/food intake measured at designated intervals. The dams were killed on GD 20 and subjected to macroscopic examination. Usual litter parameters were recorded and foetuses were sexed, weighed and submitted to external examination. About one half of the foetuses were also examined for soft tissue anomalies, and remaining foetuses were examined for skeletal anomalies.

Results
The administration of hydroxypropyl p-phenylenediamine HCl to pregnant Wistar rats over the organogenesis period produced no mortality or gross necropsy findings. Maternal toxicity occurred only at the highest dose level such as lower mean body weight, body weight gain, corrected body weight gain and food intake when compared to controls. The fetal body weight was reduced at the highest dose level. No other toxicologically significant effects on litter parameters were noted.
At the dose level of 30 mg/kg/day, the mean body weight gain, the uterine weights and the total number of live fetuses were statistically decreased when compared to controls. These decreases seem to be entirely the consequence of the small number of fetuses when compared to controls, due to an increase in pre-implantation loss. Considering that the dosing started after the implantation period, the observed effects are not considered to be related to the treatment.

Conclusion
On the basis of the results obtained in the present study, and specifically the effects on maternal and fetal body weight, the No Observed Adverse Effect Level (NOAEL) for maternal and developmental toxicity of hydroxypropyl p-phenylenediamine HCl was set at 30 mg/kg/day. Hydroxypropyl p-phenylenediamine HCl was considered to have no teratogenic potential.

Ref.: Patel, 2014

SCCS comment
The SCCS agrees that Hydroxypropyl p-phenylenediamine 2HCl does not need to be considered as a teratogen.

At 100 and 30 mg/kg/day Hydroxypropyl p-phenylenediamine 2HCl caused a decrease of maternal body weight gain, a decrease of absolute and relative uterus weight and in the number of implants. At both doses a statistically significant increase of pre-implantation loss was observed. Since dosing with A165 started only after the implantation period, the observed changes in pre-implantation loss and absolute and relative uterus weight are not related to the administration of A165.
At 30 mg/kg/day, a trend of decreased maternal body weight gain and a decreased of number of live foetuses. These trends of decreased maternal body weight gain and decreased number of live foetuses were directly related to the slight apparent increase in post-implantation loss rate. The SCCS agrees with the Applicant that the latter was related to a single dam out of 20 with a very high loss rate and that this animal should be considered as an outlier. After excluding this dam there was no difference in post implantation rate at 30 mg/kg/day compared to the control group.
In the light of the further information provided, the SCCS agrees with the Applicant that the NOAEL for maternal and developmental toxicity of hydroxypropyl p-phenylenediamine HCl was 30 mg/kg/day.

3.3.9 Toxicokinetics

3.3.9.1 Toxicokinetics in laboratory animals

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3.3.9.2 Toxicokinetics in humans

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3.3.10 Photo-induced toxicity

3.3.10.1 Phototoxicity / photo-irritation and photosensitisation

/

3.3.10.2 Photomutagenicity / photoclastogenicity

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3.3.11 Human data

/

3.3.12 Special investigations

/

3.3.13 Safety evaluation (including calculation of the MoS)

Based on an evaluation of the data provided, SCCS is of the opinion that the genotoxic potential of Hydroxypropyl p-phenylenediamine and Hydroxypropyl p-phenylenediamine 2HCl cannot be excluded. Hence, the MoS calculation is not applicable and the SCCS cannot conclude on the safety of Hydroxypropyl p-phenylenediamine and Hydroxypropyl p-phenylenediamine 2HCl.

3.3.14 Discussion

Physicochemical properties
All impurities have been quantified using either external reference standards of the corresponding impurities or area normalization. In case of the known impurities where an external reference

standard is used, the SCCS is of the opinion that a response factor should be applied for the quantitation.

Justification should be provided for the filtration of the samples using 0.2µm syringe filters prior to the HPLC analysis, as this would have removed any insoluble impurities. It should be demonstrated that the main compound and all the impurities are fully diluted in the dilution solvent prior to the HPLC analysis. The percentage recovery of the filtration procedure should be calculated by analysing samples without filtration and compare the corresponding peak areas (of the test substance and the impurities) with those obtained after filtration. HPLC-PDA chromatograms and % of all impurities) of these samples analysed with and without filtration should be comparable in terms of %area and retention times.

All impurities above 0.1% should be accurately quantified in every batch and kept at trace levels.

Detailed data on the stability of test substance in formulations (evaluated at λmax = 240 nm) should be provided.

General toxicity
The 14-day oral repeated dose toxicity study provides evidence that hydroxypropyl p-phenylenediamine 2HCl caused mortality at dose levels of 300 and 1000 mg/kg. This study indicates that the LD50 is above 300 mg/kg.

A NOAEL of 100 mg/kg/day hydroxypropyl p-phenylenediamine 2HCl was identified in the 14-day oral repeated dose toxicity study. This NOAEL is based on statistically significant decreases in red blood cell count, hematocrit and prothrombin time observed in females at 300 mg/kg bw/d. In addition, a trend increase in absolute and relative liver and kidney organ weights was observed. These changes were significant at 300 mg/kg/day.

A NOAEL of 30 mg/kg/day hydroxypropyl p-phenylenediamine 2HCl was established in the 90-day repeated dose toxicity studies. The SCCS considers the statistically significant effects in some clinical chemistry parameters as well as the statistically significant increase in relative liver weights of females at the high dose as adverse and of toxicological relevance. Hall et al (2012) suggest that in the absence of histological changes a weight-of evidence approach should be used by considering biologically significant and consistent increase in at least two further liver parameters. Since both ALT and AST levels were statistically significantly increased in the high dose females compared to controls, the SCCS considers the liver effects as adverse, resulting in a NOAEL of 30 mg/kg/day.

Hydroxypropyl p-phenylenediamine 2HCl does not need to be considered as a teratogen. A NOAEL of 30 mg/kg/day was established in the developmental toxicity study. At 100 and 30 mg/kg/day Hydroxypropyl p-phenylenediamine 2HCl caused a decrease of maternal body weight gain, a decrease of absolute and relative uterus weight and in the number of implants. At both doses a statistically significant increase of pre-implantation loss was observed. Since dosing with A165 started only after the implantation period, the observed changes in pre-implantation loss and absolute and relative uterus weight are not related to the administration of A165.
The effects observed at 30 mg/kg/day were directly related to the slight apparent increase in post-implantation loss rate. The latter was related to a single dam out of 20 with a very high loss rate, which should be considered as an outlier. After excluding this dam there was no difference in post implantation rate at 30 mg/kg/day compared to the control group.

Irritation/sensitisation
Even though hydroxypropyl p-phenylenediamine 2HCl diluted to 2% w/w in water has a very low pH=2 which may lead to corrosive effects, skin irritation was not detected in the *in vitro* Episkin study. However, the observed increase in ear thickness up to 20% in the LLNA, indicates that hydroxypropyl p-phenylenediamine 2HCl is slightly irritant to the skin.

Hydroxypropyl p-phenylenediamine 2HCl was tested in the BCOP assay, an *in vitro* method that can be used to identify ocular corrosive and severe irritants, but not mild irritants. Even though hydroxypropyl p-phenylenediamine 2HCl diluted to 2% w/w in water has a very low pH (pH=2) which may lead to corrosive effects, the BCOP assay did not indicate severe eye irritation. This, however, does not exclude mild or moderate eye irritancy potential. Under the conditions of this study, a mild to moderate eye irritation potential of the test item cannot be excluded.

The LLNA shows that hydroxypropyl p-phenylenediamine 2HCl is a moderate skin sensitiser.

Dermal absorption
The dermal absorption of hydroxypropyl p-phenylenediamine 2HCl was estimated to be 3.7 µg/cm^2 (mean + 1SD).

Mutagenicity
Mutagenicity of hydroxypropyl p-phenylenediamine (base) and its dihydrochloride salt was investigated in *in vitro* genotoxicity tests for the 3 endpoints of genotoxicity: gene mutations, chromosome aberrations (clastogenicity) and aneugenicity.

Hydroxypropyl p-phenylenediamine (base) was negative in the bacterial reverse mutation test, but positive in the *in vitro* micronucleus test in cultured human lymphocytes and is considered to be an *in vitro* mutagen.

Hydroxypropyl p-phenylenediamine 2HCl was positive in the bacterial reverse mutation test and in the *in vitro* micronucleus test. Hydroxypropyl p-phenylenediamine 2HCl was tested in the *in vitro* mammalian gene mutation test. The validity of the test provided was questioned by the SCCS, due to high variability between the experiments. Therefore, the results of this test had limited value and could not be used to exclude a genotoxic potential. Upon request of the SCCS, an additional mammalian cell gene mutation test was submitted as well as complimentary evidence from *in silico* models and a read-across approach on genotoxicity. Again, the SCCS questioned the reliability of the newly submitted mammalian gene mutation test, because the variability between the experiments was high. The additional test did not meet the acceptability criteria as described in OECD TG 476. Therefore, the SCCS considers this test as not valid and the results cannot be used to exclude a genotoxic potential for hydroxypropyl p-phenylenediamine 2HCl.

Hydroxypropyl p-phenylenediamine 2HCl did not induce cytogenetic damage leading to increased micronuclei formation in the bone marrow of rats treated orally in a 14-day repeated toxicity study up to the maximum tolerated dose of 300 mg/kg.

Supporting *in silico* and read-across data on mutagenicity were provided by the Applicant. The SCCS has noted that a systematic read-across and *in silico* approach was used and has no comments on the procedures followed. The QSAR Toolbox gave structural alerts for *in vitro* mutagenicity for both A165 and A080. Furthermore, A165 was positive according to the *in vitro* and *in vivo* predictions from the micronucleus Times model. There predictions are in line with the results obtained in the *in vitro* assays performed for A165. Although in theory, the positive results obtained from read-across, in silico modelling, or *in vitro* micronucleus test could be overruled by the negative *in vivo* micronucleus test, the SCCS cannot exclude a genotoxic potential of hydroxypropyl p-phenylenediamine in the absence of a valid mammalian gene mutation test.

Based on the data provided, the SCCS considers both the base and the salt form of hydroxypropyl p-phenylenediamine as *in vitro* mutagens and a mutagenic potential cannot be excluded.

Carcinogenicity
SCCS has noted the results of the CTA test. According to the SCCS Notes of Guidance (2016), the carcinogenic potential of a substance cannot be derived from a stand-alone CTA test.

Toxicokinetics
No data provided

Human data
No data provided

4. CONCLUSION

1. *In light of the data provided, does the SCCS consider Hydroxypropyl p-phenylenediamine and its dihydrochloride salt (A165), safe when used in oxidative hair colouring products up to a maximum on-head concentration of 2 %?*

   In light of the data provided, the SCCS considers that hydroxypropyl p-phenylenediamine and its dihydrochloride salt are not safe when used in oxidative hair colouring products due to potential genotoxicity.

2. *Does the SCCS have any further scientific concerns with regard to the use of Hydroxypropyl p-phenylenediamine and its dihydrochloride salt (A165) in cosmetic products?*

   A mild to moderate eye irritation potential of the test item cannot be excluded. Hydroxypropyl p-phenylenediamine 2HCl is a moderate skin sensitiser based on animal data.

5. MINORITY OPINION

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6. REFERENCES

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7. GLOSSARY OF TERMS

See SCCS/1602/18, 10th Revision of the SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation – from page 141

8. LIST OF ABBREVIATIONS

See SCCS/1602/18, 10th Revision of the SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation – from page 141

Appendix 1: Detailed Tables supporting the search for chemical structural analogues to A165

Table 1. Structure, similarity and Organic Functional Groups

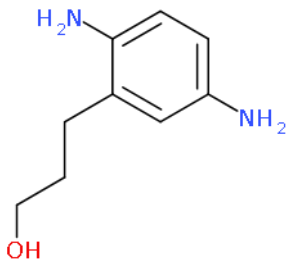
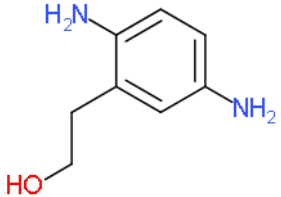
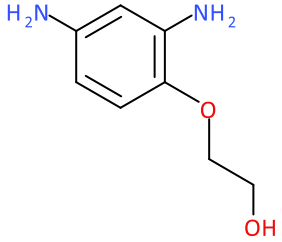
OECD QSAR TOOLBOX	**A165**	**A080**	**A042**
COMMON NAME	Hydroxypropyl p-phenylenediamine	Hydroxyethyl-p-phenylenediamine	2,4-diaminophenoxy ethanol
STRUCTURE			
SIMILARITY	100%	90-100%	70-80%
OFG	Alcohol|Amine, primary|Aniline|Aryl|Phenylenediamine, para-|Precursors quinoid compounds	Alcohol|Amine, primary|Aniline|Aryl|Phenylenediamine, para-|Precursors quinoid compounds	Alcohol|Amine, primary|Aniline|Aryl|Ether|Phenylenediamine, meta-
OFG (US EPA)	Aliphatic Carbon [CH]|Aliphatic Carbon [-CH2-]|Aliphatic Nitrogen, one aromatic attach [-N]|Aromatic Carbon [C]|Hydroxy, aliphatic attach [-OH]	Aliphatic Carbon [CH]|Aliphatic Carbon [-CH2-]|Aliphatic Nitrogen, one aromatic attach [-N]|Aromatic Carbon [C]|Hydroxy, aliphatic attach [-OH]	Aliphatic Carbon [CH]|Aliphatic Carbon [-CH2-]|Aliphatic Nitrogen, one aromatic attach [-N]|Aromatic Carbon [C]|Hydroxy, aliphatic attach [-OH]|Oxygen, one aromatic attach [-O-]
OFG (NESTED)	Alcohol|Overlapped groups|Phenylenediamine, para-|Precursors quinoid compounds	Alcohol|Overlapped groups|Phenylenediamine, para-|Precursors quinoid compounds	Alcohol|Ether|Overlapped groups|Phenylenediamine, meta-
OFG (NORBERT HAIDER)	Alcohol|Amine|Aromatic compound|Hydroxy compound|Primary alcohol|Primary amine|Primary aromatic amine	Alcohol|Amine|Aromatic compound|Hydroxy compound|Primary alcohol|Primary amine|Primary aromatic amine	Alcohol|Alkylarylether|Amine|Aromatic compound|Ether|Hydroxy compound|Primary alcohol|Primary amine|Primary aromatic amine

Table 2. Physico-chemical properties

PHYSICO-CHEMICAL PROPERTIES MODELS	**A165**	**A080**	**A042**
MOLECULAR WEIGHT (G/MOL)	166.2	152.2	168.2
CLOG P **(MODEL CLOGP VERSION V5.2)**	-0.74	-1.12	-0.87
PKA (BASE/BASE) **(MODEL ACD 12.02)**	5.868 2.912	5.933 2.977	5.014 2.857
INTRINSIC SOLUBILITY (MG/L) **(MODEL ACD 12.02)**	31272.9 (sparingly soluble)	70101.2 (soluble)	58642.3 (soluble)
SOLUBILITY (MG/L) AT **PH 3** **PH 7.4** **PH 8** **PH 9** **(MODEL ACD 12.02)**	 1000000 (freely soluble) 33030.7 (soluble) 31714.5 (sparingly soluble) 31317.1 (sparingly soluble)	 1000000 (freely soluble) 74496.6 (soluble) 71205.5 (soluble) 70211.8 (soluble)	 1000000 (freely soluble) 59003.1 (soluble) 58733 (soluble) 58651.7 (soluble)
VOLATILITY **CLASS SPICER** **CLASS PGG** **(MODEL EPISUITE V4.1)**	 Semi volatile Non volatile	 Semi volatile Non volatile	 Semi volatile Non volatile

Table 3. Mutagenicity and Genotoxicity alerts according to Times Model

MUTAGENICITY/GENOTOXICITY ALERTS (MODEL TIMES)	**A165**	**A080**	**A042**
PARENT/METABOLITE PREDICT**ED AMES MUTAGENICITY ALERT INFO**	Positive Single-ring Substituted Primary Aromatic Amines	Positive Single-ring Substituted Primary Aromatic Amines	Positive Single-ring Substituted Primary Aromatic Amines
IN VITRO **PREDICTED CHROMOSOMAL ABERRATION WITH S9** **SUMMARY MECHANISM**	Positive Interaction with DNA &Interactions with topoisomerases / proteins	Positive Interaction with DNA &Interactions with topoisomerases / proteins	Positive Interaction with DNA &Interactions with topoisomerases / proteins
IN VIVO **PREDICTED MICRONUCLEUS** **SUMMARY MECHANISM**	Positive Interaction with DNA & with topoisomerases/proteins	Positive Interaction with DNA & with topoisomerases/proteins	Negative Interaction with DNA & with topoisomerases/proteins (Detoxification path: Single-Ring Aminophenol and Alkoxyaniline Derivatives)

Table 4. Mutagenicity and Genotoxicity alerts according to OECD Toolbox Model

OECD TOOLBOX ALERT	A165	A080	A042
Carcinogenicity (genotox and nongenotox) alerts by ISS	Primary aromatic amine, hydroxyl amine and its derived esters (Genotox) Structural alert for genotoxic carcinogenicity	Primary aromatic amine, hydroxyl amine and its derived esters (Genotox) Structural alert for genotoxic carcinogenicity	Primary aromatic amine, hydroxyl amine and its derived esters (Genotox) Structural alert for genotoxic carcinogenicity
DNA alerts for AMES by OASIS	Radical mechanism via ROS formation (indirect) >> Single-Ring Substituted Primary Aromatic Amines SN1 >> Nucleophilic attack after nitrenium ion formation >> Single-Ring Substituted Primary Aromatic Amines	Radical mechanism via ROS formation (indirect) >> Single-Ring Substituted Primary Aromatic Amines SN1 >> Nucleophilic attack after nitrenium ion formation >> Single-Ring Substituted Primary Aromatic Amines	Radical mechanism via ROS formation (indirect) >> Single-Ring Substituted Primary Aromatic Amines SN1 >> Nucleophilic attack after nitrenium ion formation >> Single-Ring Substituted Primary Aromatic Amines
DNA alerts for CA and MNT by OASIS	Radical mechanism via ROS formation (indirect) >> Single-Ring Substituted Primary Aromatic Amines SN1 >> Nucleophilic attack after nitrenium ion formation >> Single-Ring Substituted Primary Aromatic Amines	Radical mechanism via ROS formation (indirect) >> Single-Ring Substituted Primary Aromatic Amines SN1 >> Nucleophilic attack after nitrenium ion formation >> Single-Ring Substituted Primary Aromatic Amines	Radical mechanism via ROS formation (indirect) >> Single-Ring Substituted Primary Aromatic Amines SN1 >> Nucleophilic attack after nitrenium ion formation >> Single-Ring Substituted Primary Aromatic Amines
DNA binding by OASIS	Radical mechanism via ROS formation (indirect) >> Single-Ring Substituted Primary Aromatic Amines SN1 >> Nucleophilic attack after nitrenium ion formation >> Single-Ring Substituted Primary Aromatic Amines	Radical mechanism via ROS formation (indirect) >> Single-Ring Substituted Primary Aromatic Amines SN1 >> Nucleophilic attack after nitrenium ion formation >> Single-Ring Substituted Primary Aromatic Amines	Radical mechanism via ROS formation (indirect) >> Single-Ring Substituted Primary Aromatic Amines SN1 >> Nucleophilic attack after nitrenium ion formation >> Single-Ring Substituted Primary Aromatic Amines
DNA binding by OECD	SN1 >> Nitrenium Ion formation >> Primary aromatic amine	SN1 >> Nitrenium Ion formation >> Primary aromatic amine	SN1 >> Nitrenium Ion formation >> Primary aromatic amine
Oncologic Primary Classification	Aromatic Amine Type Compounds	Aromatic Amine Type Compounds	Aromatic Amine Type Compounds
Protein binding alerts for Chromosomal aberration by OASIS	AN2 >> Michael addition to the quinoid type structures >> Substituted Anilines	AN2 >> Michael addition to the quinoid type structures >> Substituted Anilines	AN2 >> Michael addition to the quinoid type structures >> Substituted Anilines
***in vitro* mutagenicity (Ames test) alerts by ISS**	Primary aromatic amine, hydroxyl amine and its derived esters	Primary aromatic amine, hydroxyl amine and its derived esters	Primary aromatic amine, hydroxyl amine and its derived esters

***in vivo* mutagenicity (Micronucleus) alerts by ISS**	Primary aromatic amine, hydroxyl amine and its derived esters	Primary aromatic amine, hydroxyl amine and its derived esters	H-acceptor-path3-H-acceptor Primary aromatic amine, hydroxyl amine and its derived esters