Scientific Committee on Consumer Safety

SCCS

OPINION on
Hydroxypropyl p-phenylenediamine and its dihydrochloride salt
(A165)

(CAS/EC No. 73793-79-0/827-723-1 and 1928659-47-5/-)

The SCCS adopted this document
by written procedure on 28 February 2024

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This Opinion has been subject to a commenting period of min eight weeks after its initial publication (from 6 November to 8 January 2024). Comments received during this period were considered by the SCCS. For this Opinion, minor changes occurred in the following sections: 3.1.1.4, 3.1.5, 3.1.6, 3.1.7, title of Table 13 and reference list.

All Declarations of Working Group members are available on the following webpage:
Register of Commission expert groups and other similar entities (europa.eu)

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 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 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 in cosmetic products?

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

Keywords: SCCS, scientific opinion, Hydroxypropyl p-phenylenediamine and its dihydrochloride salt, A165, CAS/EC No. 73793-79-0/827-723-1 and 1928659-47-5/-, Regulation 1223/2009

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SCCS

The Committee shall provide Opinions on questions concerning 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 suntanning, etc.).

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1. MANDATE FROM THE EUROPEAN COMMISSION

Background

Hydroxypropyl p-phenylenediamine with the chemical name '3-(2,5-diaminophenyl)propan-1-ol' and its dihydrochloride salt with the chemical name '3-(2,5-diaminophenyl)propan-1-ol dihydrochloride salt' (CAS/EC No. 73793-79-0/827-723-1 and 1928659-47-5/-, respectively) are considered cosmetic ingredients with the reported functions of hair dyeing. Currently, they are not regulated under the Cosmetic Regulation (EC) No. 1223/2009.
In 2018, Commission services received a dossier from industry to support the safe use of Hydroxypropyl p-phenylenediamine and its dihydrochloride salt (A165) as a hair dye in cosmetic products. In its corresponding Opinion, SCCS/1608/19, the SCCS concluded that *'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'.* In addition, the SCCS noted that '*A mild to moderate eye irritation potential of the test item cannot be excluded'.*

With submission II, received in June 2022, the applicant provided additional data in order to address the issue of genotoxicity and requests to re-assess the safety of p-phenylenediamine and its dihydrochloride salt (A165) 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 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 in cosmetic products?

2. OPINION

3.1 CHEMICAL AND PHYSICAL SPECIFICATIONS

3.1.1 Chemical identity

3.1.1.1 Primary name and/or INCI name

Base
INCI name: Hydroxypropyl p-phenylenediamine

Ref: Bonnet, Analytical File, 2022

Salt
INCI name: Hydroxypropyl p-phenylenediamine HCl

Ref: Bonnet, Analytical File, 2022

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 (INCI name). Although this was not correctly named salt, the SCCS decided not to change this.

3.1.1.2 Chemical names

Base
IUPAC name: 3-(2,5-diaminophenyl)propan-1-ol

Ref: Bonnet, Analytical File, 2022

Other names: 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
IUPAC name: 3-(2,5-diaminophenyl) propan-1-ol dihydrochloride salt

Ref: Bonnet, Analytical File, 2022

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:	827-723-1	/

3.1.1.5 Structural formula

Base *Dihydrochloride salt*

3.1.1.6 Empirical formula

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

3.1.2 Physical form

Base
Light pink sometimes grey-pink powder (R0025521A)

Dihydrochloride salt:
Light beige to slightly pink powder (R0025521B)

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

Two new batches (Hydroxypropyl p-phenylenediamine HCl batch E64 and Hydroxypropyl p-phenylenediamine batch 20031802) were used in the additional safety tests included in this submission and thus these batches were also characterised.

Base:
The analytical study of R0025521A was performed on the following three batches:

- R0025521A Batch 005 D 001,

- R0025521A Batch 001 L 002,
- R0025521A Batch 010 P 002*.

* Note: R0025521A batch 10 P002 and R0025521A Batch 20031802 are the same batches. R0025521A batch 010 P002 will be used in this opinion.

Dihydrochloride salt:
The analytical study of R0025521B was performed on the following four batches:

- R0025521B Batch 004 P 001,
- R0025521B Batch 006 L 002,
- R0025521B Batch 006 L 003,
- R0025521B Batch E64.

The separation was achieved by reversed phase LC equipped with a photodiode array detector. 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:

- R0025521A 005 D 001, 001 L 002 and 010 P002 (base),
- R0025521B 004 P 001, 006 L 002, 006 L 003 and E64 (dihydrochloride salt).

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

The purity of batches:
- R0025521A 005 D 001,
- R0025521A 001 L 002,
- R0025521B 006 L 002,
- R0025521B 006 L 003,

were determined against R0025521B 004 P001 reference standard considered as 97% pure.

- The purity of batch R0025521A 010 P 002 was determined against R0025521B SCR E84-1 reference standard considered as 99.7% pure.
- The purity of R0025521B 004 P001 and of R0025521B E64 were estimated according to the formula: 100 – (impurities + solvents contents) x HPLC UV area (%) of main peak.

The purity of all tested batches is above or equal to 97% (w/w).

Table 1: Comparative table of the analytical profiles of 3 different batches of Hydroxypropyl p-phenylenediamine (R0025521A)

	Hydroxypropyl p-phenylene diamine R0025521A		
	R0025521A 005 D 001	R0025521A 001 L 002	R0025521A 010 P 002
Appearance	Light purple powder	Light pink powder	Grey to grey pink powder
IR spectrum	In accordance with the proposed structure		
UV-Vis. spectrum	Compatible with the proposed structure	*Not carried out No sample remaining*	Compatible with the proposed structure
Mass spectrum	Compatible with the proposed structure		
^{1}H NMR spectrum	In accordance with the proposed structure		
Titre HPLC (% w/w) (1)	100.7	101.0	99.5
HPLC profile *UV purity (area %)* (2)	> 99		
Impurities HPLC			
R0025495A(3) (% w/w)	Not present in the process		
R0024700A (4)	< 1000 (µg/g) *(D**)*	< 1000 (µg/g) *(D**)*	*≤ 0.03 % (ND*) (UV-Area %)*
R0011102A (5) (µg/g)	Not present in the process		
R0011103A (6) (µg/g)	Not present in the process		
R0066511A(7)	0.09 (% w/w)	< 0.1 % (UV-Area%) *(D**)*	0.05 (% w/w)
R0080941A(8) *UV purity (Area %)* (2)	0.02	≤ 0.02 *(ND*)*	≤ 0.02 *(ND*)*

**ND : Not detected – **D: Detected*

(1) Titre of batches 005 D 001 and 001 L 002 determined against 3-(2,5-diaminophenyl)propan-1-ol dihydrochloride salt, R0025521B 004 P 001, reference standard considered as pure (97 % w/w).
Estimated titre of R0025521B 004 P 001 (dihydrochloride salt):
[100-(Impurities+Solvent contents)]X UV area(%) of main peak.
Titre of batch 010 P 002 determined against R0025521B batch SCR E84-1 reference standard considered as pure (99.7 % w/w).
(2) UV detection*: UV purity* - Area %, without response factor.
Irrespective of residual solvents, salts and other non-detectable products λ = 230-700 nm
(3) R0025495A: *2-propylbenzene-1,4-diamine dihydrochloride salt.*
(4) R0024700A: *1,2,3,4-tetrahydroquinolin-6-amine dihydrochloride salt, against R0024700A 001 L 001 reference standard considered as pure (69 % w/w). (Note: R0024700C is the same molecule). UV-Area % for R0025521A 010 P 002*
(5) R0011102A: *4-aminophenylamine dihydrochloride salt.*
(6) R0011103A: *2-methylbenzene-1,4-diamine dihydrochloride salt.*
(7) R0066511A: *Quinoline-6 amine,*
In R0025521A 005 D 001, impurity content determined against R0066511A 001 L 001 reference standard considered as pure (99.8 % w/w).
In R0025521A 001 L 002, UV purity - Area %, without response factor.
In R0025521A 010 P002, impurity content determined against R0025521B 001 SCR1 SCR E84-1 (main product), reference standard considered as pure (99.7 % w/w).
(8) *6-amino-3,4-dihydroquinolin-2(1H)-one*

Table 2: Comparative table of the analytical profiles of 4 different batches of Hydroxypropyl p-phenylenediamine, dihydrochloride salt (R0025521B).

	Hydroxypropyl p-phenylene diamine, 2 HCl R0025521B			
	R0025521B 004 P 001	R0025521B 006 L 002	R0025521B 006 L 003	R0025521B E64
Appearance	Light beige powder			Light beige powder, slightly pink
IR spectrum	In accordance with the proposed structure			
UV-Vis. spectrum	Compatible with the proposed structure			
Mass spectrum	Compatible with the proposed structure			
^{1}H NMR spectrum	In accordance with the proposed structure			
HPLC Titre (% w/w)	97 *(Estimated titre***)*	101.2±1.6 [1]	99.8±1.4 [1]	99.4 *(Estimated titre***)*
HPLC profile UV purity (area %) [2]	> 95			
Impurities HPLC				
R0025495A [3] (% w/w)	0.79	Not present in the process		
R0024700A [4] (µg/g)	< 1000 (D**)			≤ 300 *(ND*)* *(UV-Area %)*
R0011102A [5] (µg/g)	< 40 (D**)	< 40 (ND*)		Not present in the process
R0011103A [6] (µg/g)	< 50 (D**)	< 50 (ND*)		Not present in the process
R0066511A [7]	< 0.1 (ND*) (UV- Area %)	< 0.1 (D**) (UV- Area %)		0.03 (%w/w)
R0080941A [8] (UV-area %) [2]	≤ 0.01 *(ND*)*	≤ 0.01 *(ND*)*	0.01	≤ 0.02 *(ND*)*

**ND: Not detected – **D: Detected*

****Estimated titre of R0025521B (dihydrochloride salt): [100-(Impurities+Solvent contents)]X UV area(%) of main peak.*

(1) Titre of batches 006 L 002 and 006 L 003 determined against 3-(2,5-diaminophenyl)propan-1-ol dihydrochloride salt, R0025521B 004 P 001, reference standard considered as pure (97% w/w).

Titre of batch E64: Estimated titre (See above).

(2) UV detection: UV purity - Area %, without response factor. Irrespective of residual solvents, salts and other non-detectable products λ = 230-700 nm

(3) R0025495A: 2-propylbenzene-1,4-diamine dihydrochloride salt, content determined against R0025495A 001 L001 reference standard considered as pure (99.9 % w/w).

(4) R0024700A: 1,2,3,4-tetrahydroquinolin-6-amine dihydrochloride salt, content determined against R0024700A 001 L 001 reference standard considered as pure (69 % w/w). (Note: R0024700C is the same molecule).

UV-Area % for R0025521B E64
(5) R0011102A: 4-aminophenylamine dihydrochloride salt, content determined against R0011102A 000 L 139 reference standard considered as pure (99.8 % w/w)

(6) R0011103A: 2-methylbenzene-1,4-diamine dihydrochloride salt, content determined against R0011103A 001 L 001 reference standard considered as pure (99.3 % w/w)

(7) R0066511A: Quinoline-6 amine,

In R0025521B batches 004 P 001,006 L 002 and 006 L 003, UV purity - Area %, without response factor.

In R0025521B batch E64, impurity content determined against R0025521B batch SCR E84-1(main product) reference standard considered as pure (99.7 % w/w).

(8) 6-amino-3,4-dihydroquinolin-2(1H)-one

Ref: Bonnet B. Analytical file, 2022

3.1.5 Impurities / accompanying contaminants

Potential impurities for R0025521 A/B can be starting material, synthesis intermediaries, by-products or residual solvents (Table 3).

Table 3: Chemical structures of R0025521 A/B and their potential impurities

Code Chloe	Structure
R0025521A (base) $C_9H_{14}N_2O$: 166.22 R0025521B (dihydrochloride salt) $C_9H_{14}N_2O, 2HCl$: 239.14	NH₂, OH, 2HCl
R0025495A $C_9H_{14}N_2, 2HCl$: 223.15	NH₂, NH₂, 2HCl
R0078106A $C_9H_{12}N_2O_3$: 196.21	NH₂, OH, O⁻ N⁺ O
R0078355A C_9H_9NO : 147.18	O, HN
R0078356A $C_9H_8N_2O_3$: 192.18	O, HN, O⁻ N⁺ O
R0024700A $C_9H_{12}N_2, 2HCl$: 221.13	NH, NH₂, 2HCl
R0011102A $C_6H_8N_2, 2HCl$: 181.06	NH₂, NH₂, 2HCl
R0011103A $C_7H_{10}N_2, 2HCl$: 195.09	NH₂, NH₂, 2HCl
R0066511A $C_9H_8N_2$: 144.18	N, NH₂
R0080941A $C_9H_{10}N_2O$: 162.19	O, HN, NH₂

By using HPLC-PDA analysis, R0025521A (base) or R0025521B (dihydrochloride salt) was mainly detected; its retention time is around 6.4 minutes.

Several impurities were detected:
- One of them was identified as R0025495A (Rt = 19.9min) in R0025521B (dihydrochloride salt) Batch 004 P 001. R0025495A content was determined using an external calibration against a reference standard R0025495A batch 001 L 001.

• synthesis intermediates were not detected in R0025521B (dihydrochloride salt) Batches 006 L 002 and 006 L 003
• R0066511A was detected with a level lower than 0.1% (%area) in R0025521A (base) Batch 001 L 002 and in R0025521B (dihydrochloride salt) Batches 006 L 002 and 006 L 003.
In R0025521B (dihydrochloride salt) Batch 004 P 001, R0066511A was not detected.
In R0025521A (base) Batch 005 D 001, R0066511A was detected with a level greater than 0.1% (% area). So, R0066511A content is determined using an external calibration against a reference standard R0066511A 001 L 001.

Other impurities were also detected and their relative % area are given in the following tables.

Table 4: Impurities of R0025521 A (base) in 3 batches

Impurities	R0025521A (base)		
	R0025521A 005 D 001	R0025521A 001 L 002	R0025521A 010 P 002
	% Area (λ : 230 – 700 nm)		
9.3 min (Imp. 1)	0.05	ND	≤ 0.02 ND
11 min (Imp. 5) R0080941A	0.02	ND	ND
12.8 min (Imp. 2)	0.05	0.08	ND
Imp. 6 : R0024700A	Specific method was developed		
18 min (Imp. 3)	0.04	0.06	0.07
19.1 min (Imp. 4) R0066511A	0.24	0.01	0.18
19.8 min (Imp. 7) R0025495A	Not generated in this process		
22.2 min (Imp. 8) R0078355A	ND	ND	ND
22.5 min (Imp. 9) R0078106A	ND	ND	ND
23.0 min (Imp. 10) R0078356A	ND	ND	ND

*ND: not detected

In green: Impurities with a level greater than 0.1% (% area), to be quantified.
In red: a specific method was developed for R0024700A.
An analytical approach for the elucidation of the impurities with a level greater than 0.1% (% area) was performed.

Table 5: Impurities of R0025521 B (base) in 3 batches

Impurities	R0025521B (dihydrochloride salt)			
	R0025521B 004 P 001	R0025521B 006 L 002	R0025521B 006 L 003	R0025521B E64
	% Area (λ : 230 – 700 nm)			
9.3 min (Imp. 1)	ND	ND	ND	ND
11 min (Imp. 5) R0080941A	ND	ND	0.01	≤ 0.02 ND
12.8 min (Imp. 2)	0.08	0.11	0.11	ND
Imp. 6 : R0024700A	Specific method was developed			
18 min (Imp. 3)	0.19	0.06	0.07	0.05
19.1 min (Imp. 4) R0066511A	ND	0.05	0.06	0.17
19.8 min (Imp. 7) R0025495A	0.59	ND	ND	ND
22.2 min (Imp. 8) R0078355A	ND	ND	ND	ND
22.5 min (Imp. 9) R0078106A	ND	ND	ND	ND
23.0 min (Imp. 10) R0078356A	ND	ND	ND	ND

ND : Not Detected

In green: Impurities with a level greater than 0.1% (% area), to be characterised (and quantified if possible - No reference for Imp.2).
In red: a specific method was developed for R0024700A.
An analytical approach for the elucidation of the impurities with a level greater than 0.1% (% area) was performed.

Ref: Bonnet, Analytical file Annex 6, 2022

Evaluation of R0025495A content

Determination by HPLC against a reference standard R0025495A 001 L 001 considered as pure (99.9%).

- **R0025495A** content in R0025521B (dihydrochloride salt) Batch 004 P 001: 0.79 % w/w

Note: R0025495A cannot be present in the process of the other batches R0025521A or R0025521B.

Evaluation of R0011102A and R0011103A contents

Determination by HPLC against:
- Reference standard R0011102A 000 L 139 considered as pure (99.8%).
- Reference standard R0011103A 001 L 001 considered as pure (99.3%).

- **R0011102A** content in:

- R0025521B (dihydrochloride salt) 004 P 001: < 40 µg/g Detected
- R0025521B (dihydrochloride salt) 006 L 002: < 40 µg/g Not detected
- R0025521B (dihydrochloride salt) 006 L 003: < 40 µg/g Not detected

Note: R0011102A cannot be present in the process of R0025521A (base) batches.

Ref: Bonnet, Analytical file Annex 8, 2022

- **R0011103A** content in:

- R0025521B (dihydrochloride salt) 004 P 001: < 50 µg/g Detected
- R0025521B (dihydrochloride salt) 006 L 002: < 50 µg/g Not detected
- R0025521B (dihydrochloride salt) 006 L 003: < 50 µg/g Not detected

Note: R0011103A cannot be present in the process of R0025521A (base) batches. R0011102A and R001103A cannot be present in the process of R0025521B batch E64. R0025521 batch E64 was chloridated from a batch of R0025521A, which cannot contain the 2 impurities R0011102A and R0011103A.

Ref: Bonnet, Analytical file, Annex 8, 2022

Evaluation of R0066511A content
Determination by HPLC:

- **R0066511A** content in:

- R0025521A (base) 005 D 001: 0.09 % w/w
against reference standard R0066511A 001 L 001 considered as pure (99.9%).
- R0025521A (base) 001 L 002: 0.01% (UV- Area%)
- R0025521A (base) 010 P 002: 0.05% w/w
against reference standard R0025521B 001 SCR E84-1(main product) considered as pure (99.9%).

- R0025521B (dihydrochloride salt) 004 P 001: < 0.1% (UV- Area%) Not detected
- R0025521B (dihydrochloride salt) 006 L 002: < 0.1% (UV- Area%) Detected
- R0025521B (dihydrochloride salt) 006 L 003: < 0.1% (UV- Area%) Detected
- R0025521B (dihydrochloride salt) batch E64: 0.03% w/w
against reference standard R0025521B 001 SCR E84-1(main product) considered as pure (99.9%).

Ref: Bonnet, Analytical file, Annex 9, 2022

Evaluation of R0024700A content
Determination by HPLC against a reference standard R0024700A 001 L 001 considered as pure (69%). For R0025521A 010 P 002 and R0025521B E64: UV-Area %.

- **R0024700A** content in:

- R0025521A (base) 005 D 001: < 0.1% (% w/w) Detected
- R0025521A (base) 001 L 002: < 0.1% (% w/w) Detected
- R0025521A (base) 010 P 002: < 0.03% (UV-Area %) Not detected
- R0025521B (dihydrochloride salt) 004 P 001: < 0.1% (% w/w) Detected
- R0025521B (dihydrochloride salt) 006 L 002: < 0.1% (% w/w) Detected
- R0025521B (dihydrochloride salt) 006 L 003: < 0.1% (% w/w) Detected
- R0025521B (dihydrochloride salt) batch E64: < 0.03% (UV-Area %) Not detected

(Note: R0024700A and R0024700C are the same molecule).

Ref: Bonnet, Analytical file, Annex 10, 2022

Structural identification of unknown impurities:

Chemical structures were proposed for unknown detected impurities with a content above 0.1% (UV-Area %).

Four impurities were detected in the various batches at a level greater than 0.1% (except impurity N°1) as determined from the UV-visible profile. These impurities have been analysed by HPLC/DAD/ESI-MS and their structures are presented in Table 6.

Table 6: Structural formulas of the four impurities detected

Impurity number and description	Structural formula
Impurity N°1: 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**	O, HN, NH$_2$ 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**	NH, OH, NH 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**.	N, NH Exact Mass =146.08440 Molecular Formula =C9H10N:
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**	N, H$_2$N Exact Mass =144.06875 Molecular Formula =C9H8N:

Ref: Analytical file Annex 11, 2014; Bonnet, Analytical file Annex 11, 2022

Residual solvents

Base:
Residual solvents contents for R0025521A (base) were evaluated by NMR except for batch 010 P002 (HS-GC).

R0025521A batch 005 D001:
- Ethanol: # 0.002 M/Mole (# 0.06 % w/w) – NMR

R0025521A batch 001 L 002:
- Methanol: < 0.005 M/Mole (< 0.1% w/w) – NMR

R0025521A batch 010 P 002:

- Ethyl acetate: 100 µg/g – HS-GC
- Methyl tert-butyl ether: 100 µg/g – HS-GC

Ref: Bonnet, Analytical file Annex 3, 2022

Dihydrochloride salt:
Residual solvents contents for R0025521B (dihydrochloride salt) were carried out by HS-GC.

R0025521B batch 004 P 001
- Ethanol: 2600 µg/g
- Ethyl ether 500 µg/g
- Chloroethane: 500 µg/g
- Methylethylether: 50 µg/g

R0025521B batch 006 L 002
- Ethanol: <1000 µg/g
- Ethyl ether: <100 µg/g
- Chloroethane: 20 µg/g

R0025521B batch 006 L 003
- Ethanol: < 1000 µg/g
- Ethyl ether: < 100 µg/g
- Chloroethane: 30 µg/g

R0025521B batch E64
- Ethanol: 0.2 % w/w
- Methyl tert-butyl ether: 0.01 % w/w
- iso-propyl alcohol: 0.2 % w/w

The solvents detected may be different due to some differences in the process of the different batches.

Ref: Bonnet, Analytical file Annex 12, 2022

Determination of ash content
This determination was performed for R0025521A (base) batches 005 D 001, 001 L 002 and R0025521B (dihydrochloride salt) batches 004 P 001, 006 L 002, 006 L 003 and E64.
Results:
- R0025521A Batch 005 D 001: < 0.1% w/w
- R0025521A Batch 001 L 002: 0.49% w/w
- R0025521A Batch 010 P 002: Not determined
- R0025521B Batch 004 P 001: < 0.1% w/w
- R0025521B Batch 006 L 002: < 0.1% w/w
- R0025521B Batch 006 L 003: 0.2% w/w
- R0025521B Batch E64: < 0.1% w/w

Table 7: Elemental analysis

Elemental analysis was carried out as follows:
- C, H and N: Thermal conductivity
- O: Infrared

▪ R0025521A (**base**):

	Theoretical Values (% w/w)	R0025521A (base) Experimental values (% w/w)		
		005 D 001	001 L 002	010 P 002
Carbon	65.0	65.1	65.0	65.3
Hydrogen	8.5	8.5	8.4	8.5
Oxygen	9.6	9.5	9.6	9.6
Nitrogen	16.9	16.7	16.8	16.9

R0025521B (**dihydrochloride salt**):

	Theoretical Values (*% w/w*)	R0025521B (dihydrochloride salt) Experimental values (*% w/w*)			
		004 P 001	006 L 002	006 L 003	E64
Carbon	45.2	45.6	45.4	45.1	45.1
Hydrogen	6.7	6.7	6.8	6.7	6.7
Oxygen	6.7	7.0	6.6	6.8	7.6
Nitrogen	11.7	11.6	11.7	11.6	11.6

Chloride content

Determination of chloride content was carried out by potentiometry.

	Theoretical Values (*% w/w*)		R0025521 A (base) Experimental values (*% w/w*)			R0025521 B (dihydrochloride salt) Experimental values (*% w/w*)			
			005 D 001	001 L 002	010 P 002	004 P 001	006 L 002	006 L 003	E64
Chloride	(A) 0	(B) 29.7	-	-	-	29.3	29.4	30.4	28.9

Determination of heavy metals content
Determination of metal contents by ICP/AES, ICP-MS or atomic fluorescence after mineralisation (Hg).

R0025521A **(base)**

Metal	R0025521A (base) Experimental values (mg/kg)		
	Batch 005 D 001	Batch 001 L 002	Batch 010 P 002
Aluminium	13	26	<5
Antimony	<1	<1	<1
Arsenic	<1	<1	<1
Baryum	<5	<5	<5
Calcium	65	67	<50
Cadmium	<1	<1	<1
Cobalt	<5	<5	<5
Chromium	<5	<5	<5
Copper	<5	<5	<5
Iron	<5	<5	<5
Mercury	<1	<1	<1
Potassium	<50	<50	<50
Manganese	<5	<5	<5
Molybdenum	<5	<5	<5
Sodium	<50	<50	<50
Nickel	<5	<5	<5
Phosphorous	<50	<50	<50
Lead	<1	<1	<1
Palladium	<1	<1	<1
Selenium	<5	<5	<5
Tin	<5	<5	<5
Titan	<5	<5	<5
Vanadium	<5	<5	<5
Zinc	<5	<5	<5

R0025521B **(dihydrochloride salt)**

Metal	R0025521B (dihydrochloride salt) Experimental values (mg/kg)			
	Batch 004 P 001	Batch 006 L 002	Batch 006 L 003	Batch E64
Aluminium	<5	<5	11	<5
Antimony	<1	<1	<1	<1
Arsenic	<1	<1	<1	<1
Baryum	<5	<5	<5	<5
Calcium	<50	<50	557	<50
Cadmium	<1	<1	<1	<1
Cobalt	<5	<5	<5	<5
Chromium	<5	<5	<5	<5
Copper	<5	<5	<5	<5
Iron	15	<5	7	<5
Mercury	<1	<1	<1	<1
Potassium	<50	<50	<50	<50
Manganese	<5	<5	<5	<5
Molybdenum	<5	<5	<5	<5
Sodium	<50	<50	<50	<50
Nickel	<5	<5	<5	<5
Phosphorous	267	270	311	50
Lead	<1	<1	<1	<1
Palladium	3	1	<1	<1
Selenium	<5	<5	<5	<5
Tin	<5	<5	<5	<5
Titan	<5	<5	<5	<5
Vanadium	<5	<5	<5	<5
Zinc	<5	<5	<5	<5

SCCS comment

- Impurities at content above 0.1% have been quantified using either external reference standards of the corresponding impurities or area normalisation. According to the Applicant, in case of the known impurities where an external reference standard is used a response factor was applied for the quantitation.
- It should be demonstrated that the main compound and all the impurities are fully dissolved in the dilution solvent prior to the HPLC analysis.
- All impurities above 0.1% should be accurately quantified in every batch; effort should be made during the manufacturing procedure to keep these impurities 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

Solubility in water (According to OECD 105 – shake-flask method): > 486 g/L (Test carried out on R0025521A 005 D 001); data generated by Ibacon.

Ref: Bonnet, Analytical file, 2022

According to the Applicant, prior to starting safety studies, the solubility of A165 in the most appropriate solvents was conducted and thus, no solubility issue is expected while running the safety tests.

From previous Opinion (SCCS/1608/19)
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

Solubility in water (According to OECD 105 – shake-flask method): > 486 g/L (Test carried out on R0025521B 004 P 001); data generated by Ibacon.

Ref: Bonnet, Analytical file, 2022

3.1.7. Partition coefficient (log Pow)

Base:
During the commenting period the Applicant has submitted additional data on the LogPow value of the base form (R0025521A batch 20031802) which was found to be equal to 0.4 by an HPLC method. Because the retention time of the test item was outside the range of retention times obtained for the reference items, a log Pow limit value of < 0.5 is stated.

Ref: 131461186_Report_logPowHPLC_20190121_Final_signed

Dihydrochloride salt:
Measured on R0025521B 004 P 001 (dihydrochloride salt):
- Log Po/w Calculated: -0.70 (ClogP, v5.2) code error 0.012
- Log Po/w Experimental: -0.60 (Determination by potentiometry)

SCCS comment
The developed HPLC method for the calculation of LogPow value for the base form of the test substance is not accurate because the test item was outside the range of retention times. Therefore, it only provides an estimation of LogPow.

3.1.8 Additional physical and chemical specifications

From previous Opinion (SCCS/1608/19)

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/Vis absorption, 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 8: UV-Vis spectra of R0025521A (base)

	R0025521A (base) Batch 005 D 001	R0025521A (base) Batch 010 P 002
Concentration (in water)	# 0.0005 % w/w	# 0.0004 % w/w
Absorbance (λ=200nm) **E1% - 1cm**	1.058 2116	0.799 1997.5
Absorbance (λ=240nm) **E1% - 1cm**	0.245 490	0.183 457.5
Absorbance (λ=290nm) **E1% - 1cm**	0.062 124	0.045 112.5

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 9: UV-Vis spectra of ROO25521B (dihydrochloride) batches 004 P001, 006 L002, 006 L003, E64.

	R0025521B (2 HCl) Batch 004 P 001	R0025521B (2 HCl) Batch 006 L 002	R0025521B (2HCl) Batch 006 L 003	R0025521B (2HCl) Batch E64
Concentration (in water)	# 0.0004 %			
Absorbance (λ=200nm) **E1% - 1cm**	0.808 2020	0.700 1750	0.643 1607.5	0.667 1667.5
Absorbance (λ=240nm) **E1% - 1cm**	0.148 370	0.127 317.5	0.117 292.5	0.121 302.5
Absorbance (λ=290nm) **E1% - 1cm**	0.034 85	0.028 70	0.025 62.5	0.023 57.5

UV-vis spectra of all tested batches (base and dihydrochloride salt) were comparable.

3.1.9 Homogeneity and Stability

From previous Opinion (SCCS/1608/19)

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, 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 hours after preparation.

Ref: Gohel, 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 4oC 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 4oC 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 storing 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 TOXICOKINETIC

3.2.1 Dermal / percutaneous absorption

From previous Opinion (SCCS/1608/19)

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

3.2.2 Other studies on toxicokinetics

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3.3 EXPOSURE ASSESSMENT

3.3.1 Function and uses

From previous opinion (SCCS/1608/19)

The ingredients hydroxypropyl p-phenylenediamine and its dihydrochloride salt (A165) are intended to be used in oxidative hair colouring products at on-head concentrations 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 they are 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 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, regardless of which initial form (salt or free base) of the ingredient is used to formulate the product.

3.3.2 Calculation of SED/LED

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3.4 TOXICOLOGICAL EVALUATION

3.4.1. Irritation and corrosivity

3.4.1.1 Skin irritation

From previous Opinion (SCCS/1608/19)
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.

3.4.1.2 Mucous membrane irritation / eye irritation

From previous opinion (SCCS/1608/19)

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.

3.4.2 Skin sensitisation

From previous Opinion (SCCS/1608/19)

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.

3.4.3 Acute toxicity

3.4.3.1 Acute oral 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.

3.4.3.2 Acute dermal toxicity

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3.4.3.3 Acute inhalation toxicity

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3.4.4 Repeated dose toxicity

From previous Opinion (SCCS/1608/19)

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 that were 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.

3.4.4.1 Repeated dose (28 days) oral / dermal / inhalation toxicity

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

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3.4.4.3 Chronic (> 12 months) toxicity

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3.4.5 Reproductive toxicity

3.4.5.1 Fertility and reproduction toxicity

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3.4.5.2 Developmental Toxicity

From previous Opinion (SCCS/1608/19)

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.

3.4.6 Mutagenicity / genotoxicity

The safety profile of hydroxypropyl p-phenylenediamine and its dihydrochloride salt was evaluated by SCCS in 2019 (SCCS/1608/19 Final Opinion). At that time, the SCCS concluded that "hydroxypropyl p-phenylenediamine and its dihydrochloride salt are not safe when used in oxidative hair colouring products due to potential genotoxicity".

SCCS overall comment on mutagenicity (SCCS/1608/19 Final Opinion)
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*. The Table below 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 10. Summary of the genotoxicity data in Opinion on Hydroxypropyl p-phenylenediamine and its dihydrochloride salt (A165) (SCCS/1608/19 Final Opinion).

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

In the present Opinion the following studies have been submitted: the reconstructed skin micronucleus assay (RSMN) with the base form, *in vitro* alkaline Comet assay in reconstructed 3D full thickness human skin model with the dihydrochloride form, and ToxTracker with both forms. The Applicant submitted analytical dossier.

Additionally, a new mammalian gene mutation test was submitted upon request from the SCCS on Hydroxypropyl p-phenylenediamine 2HCl, because in the absence of a valid mammalian gene mutation test, the SCCS could not exclude a genotoxic potential of hydroxypropyl p-phenylenediamine'.

3.4.6.1 Mutagenicity / genotoxicity *in vitro*

Newly submitted genotoxicity studies

Hydroxypropyl p-phenylenediamine (base form)

***In Vitro* Micronucleus Test using Reconstructed skin Micronucleus (RSMN) assay in EpiDermTM**

The purpose of this study was to evaluate the genotoxic potential of the test article R0025521A, by assessing induction of micronuclei in the reconstructed skin micronucleus assay (RSMN) in EpiDermTM

Test Material
Hydroxypropyl p-phenylenediamine (commercial name IMEXINE OBN) batch 20031802 (HPLC purity 99.8%) was used in this study.

Test System
EpiDermTM tissue from the MatTek Corporation (Ashland, MA, USA) was used in this experiment. The EpiDermTM model is a multilayered, differentiated tissue consisting of basal, spinous, granular and cornified layers resembling the normal human epidermis. This system has been demonstrated to be sensitive to the clastogenic and aneugenic activity of variety of chemicals.

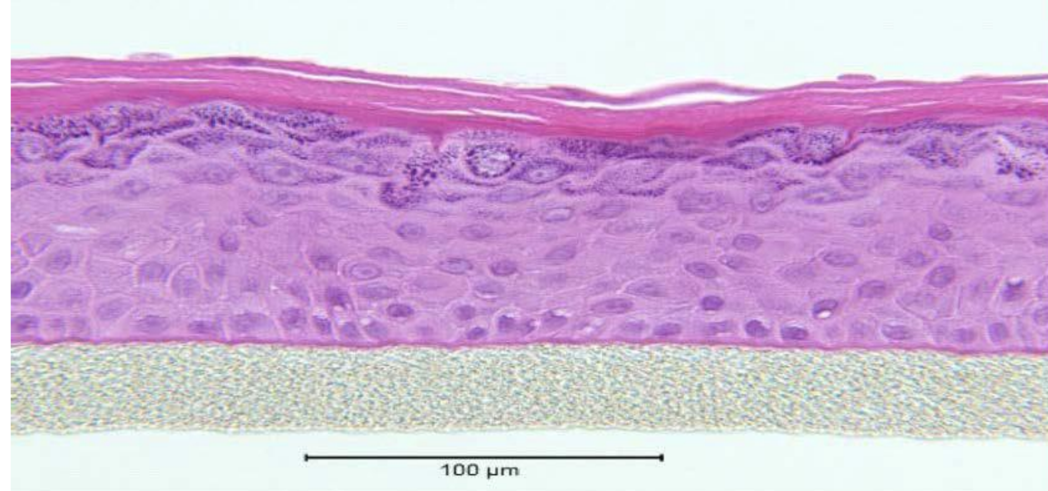

Figure 1: Sectional view of 3D skin tissue showing intact multilayer of skin and basal layer of actively dividing keratinocytes (for details refer Curren et al., 2006).

Test Procedure

Tissues were treated by application of 10 µL of the test article/vehicle mixture at the appropriate concentration on the top surface of the EpiDermTM tissue. In the preliminary cytotoxicity and the definitive micronucleus assays, EpiDermTM tissues were treated twice, 24 hours apart and tissues were processed at 48 hours (2-day dosing regimen). In the confirmatory micronucleus assay, the tissues were treated three times, 24 hours apart and tissues were processed at 72 hours (3-day dosing regimen).

The preliminary cytotoxicity test was conducted by exposing a single tissue per concentration to vehicle alone and 15 concentrations of the test article ranging from 0.006 to 100 mg/mL.

Based on these results, the definitive micronucleus assay was conducted using triplicate tissues at 6 concentrations of the test article ranging from 0.031 to 25.5 mg/mL. In the definitive micronucleus assay, ≥ 50% cytotoxicity by calculating CBPI and RVCC relative to vehicle control was observed at the concentrations of ≥ 12.5 mg/mL. Precipitate was observed on the tissue at concentrations ≥ 6.25 mg/mL at the end of second treatment. The concentrations selected for scoring micronuclei were 0.313, 3.13, and 6.25 mg/mL

One thousand binucleated cells per tissue were scored for the presence of micronuclei.

Since the result of the micronucleus assay using a 2-day dosing regimen was negative, a confirmatory assay was conducted with a 3-day dosing regimen at concentrations ranging from 0.016 to 12.5 mg/mL using triplicate tissues. The highest dose evaluated for micronuclei was selected based on visible precipitate observed on the tissue at concentrations ≥ 3.13 mg/mL at the end of the third treatment.

The concentrations selected for scoring micronuclei were 0.031, 0.313, and 3.13 mg/mL.
The slides were stained with acridine orange. A minimum of 1000 binucleated cells with intact and red-stained cytoplasm were analysed per tissue.

Cytotoxicity was determined for each model by calculating the cytokinesis-blocked proliferation index (CBPI) and by the relative viable cell count (RVCC).
Negative (Acetone) and positive controls (Mitomycin C MMC, Carbendazim CBZ, Cyclophosphamide CP) were run in parallel.
Test article dilutions were prepared immediately before use and delivered to the test system at room temperature under filtered light.
The study was conducted under GLP.

Results
In the definitive micronucleus assay, the percentage of micronucleated binucleated cells in the vehicle control was within the acceptable historical control range and the percentage of micronucleated binucleated cells in the positive control was statistically increased and within the historical positive range.

The percentage of cells with micronucleated binucleated cells in the test article-treated tissues was not significantly increased relative to the vehicle control at any concentration, and no significant dose response trend was observed. The percentage of micronucleated cells in the MMC and CBZ was statistically significant relative to the vehicle control.
In the confirmatory micronucleus assay, the percentage of micronucleated binucleated cells in the vehicle control was within the acceptable historical control range and the percentage of micronucleated binucleated cells in the positive control was statistically increased and within the historical positive range.

The percentage of cells with micronucleated binucleated cells in the test article-treated tissues was not significantly increased relative to the vehicle control at any concentration, and no significant dose response trend was observed. The percentage of micronucleated cells in the CBZ and CP was statistically significant relative to the vehicle control.

Table 11. Summary of the results on micronucleus assay: Evaluation in EpiDerm™ 3D skin treated with R0025521A

Micronucleus Assay: Micronucleus Evaluation in EpiDerm™ 3D Skin Treated with R0025521A Summary

Treatment (mg/mL)	Tissue No.	BN Counted	MNBN Counted	%MNBN	%MNBN per Dose	
					Mean	SD
Acetone	1	1000	0	0.00	0.10	0.10
	2	1000	1	0.10		
	3	1000	2	0.20		
0.313	7	1000	1	0.10	0.03	0.06
	8	1000	0	0.00		
	9	1000	0	0.00		
3.13	10	1000	1	0.10	0.10	0.00
	11	1000	1	0.10		
	12	1000	1	0.10		
6.25 p	13	1000	1	0.10	0.10	0.10
	14	1000	2	0.20		
	15	1000	0	0.00		
CBZ.0.75	25	1000	19	1.90	1.97**	0.50
	26	1000	15	1.50		
	27	1000	25	2.50		
MMC, 6µg/mL	31	1000	11	1.10	1.40**	0.26
	32	1000	16	1.60		
	33	1000	15	1.50		

BN = Binucleated cells
MNBN = Micronucleated binucleated cells
SD=Standard Deviation
** $p \leq 0.01$, Fisher's Exact Test, relative to the vehicle control
p = precipitation observed on tissue at the end of last treatment

Table 12. Summary of the results from micronucleus assay (confirmatory assay): Evaluation in EpiDerm™ 3D skin treated with R0025521A

Confirmatory Assay: Micronucleus Evaluation in EpiDerm™ 3D Skin Treated with R0025521A Summary

Treatment (mg/mL)	Tissue No.	BN Counted	MNBN Counted	%MNBN	%MNBN per Dose	
					Mean	SD
Acetone	1	1000	1	0.10	0.13	0.06
	2	1000	2	0.20		
	3	1000	1	0.10		
0.031	7	1000	1	0.10	0.13	0.06
	8	1000	1	0.10		
	9	1000	2	0.20		
0.313	10	1000	2	0.20	0.20	0.10
	11	1000	1	0.10		
	12	1000	3	0.30		
3.13 p	13	1000	3	0.30	0.17	0.12
	14	1000	1	0.10		
	15	1000	1	0.10		
CBZ, 0.125	28	1000	13	1.30	1.50**	0.20
	29	1000	17	1.70		
	30	1000	15	1.50		
CP, 60	25	1000	9	0.90	1.10**	0.26
	26	1000	10	1.00		
	27	1000	14	1.40		

Conclusion
Under the conditions of the study, hydroxypropyl p-phenylenediamine was concluded to be negative for the induction of micronuclei in the reconstructed skin micronucleus assay (RSMN) in EpiDermTM.

Roy, 2020

SCCS comment
The SCCS acknowledges that the Reconstructed skin Micronucleus (RSMN) assay is a promising test to be used as second tier assay to follow-up on positive results from standard *in vitro* assays for dermally applied compounds even if RSMN is not adopted yet as an OECD TG. The SCCS agrees that hydroxypropyl p-phenylenediamine is negative for the induction of micronuclei in this assay.

ToxTracker®

ToxTracker® is a newly developed test that explores mechanistic insight on genotoxicity and can accurately classify compounds as genotoxic or non-genotoxic. ToxTracker is a panel of mammalian stem cell lines that contain different fluorescent reporters for induction of DNA damage, oxidative stress, and protein damage. The test substance was analysed in absence and presence of an S9 rat liver extract-based metabolising system.
Although the experiments were conducted as a non-GLP study, general principles to conduct proper scientifically correct *in vitro* experiments were adhered to. For all ToxTracker analyses, test facility strictly followed the OECD Good Cell Culture Practice guidelines.

Test Materials
Hydroxypropyl p-phenylenediamine batch 001L002 were used in this study.

Test System
The ToxTracker assay is a panel of six validated green fluorescent protein (GFP)-based mouse embryonic stem (mES) reporter cell lines representing four distinct biological responses that are associated with carcinogenesis, i.e., general cellular stress, DNA damage, oxidative stress and the unfolded protein response. The induction of GFP reporters by test compounds is efficiently measured across a broad concentration range using flow cytometry, resulting in quantitative data on dose-response relationships.

In contrast to the cancer-derived cell lines that are currently used for *in vitro* genotoxicity testing, stem cells are genetically stable and proficient in all cellular pathways required for accurate detection of potentially carcinogenic properties of compounds. Extensive whole-genome transcription profiling has led to identification of a panel of biomarker genes that are preferentially activated upon exposure to different classes of carcinogens and toxicants.

The ToxTracker assay contains two reporters for genotoxicity: Bscl2-GFP, which is activated upon formation of bulky DNA lesions and subsequent DNA replication stress and Rtkn-GFP, which is activated upon induction of DNA double strand breaks.

ToxTracker is a mammalian stem cell-based assay that monitors activation of specific cellular signaling pathways for detection of the biological reactivity of compounds.

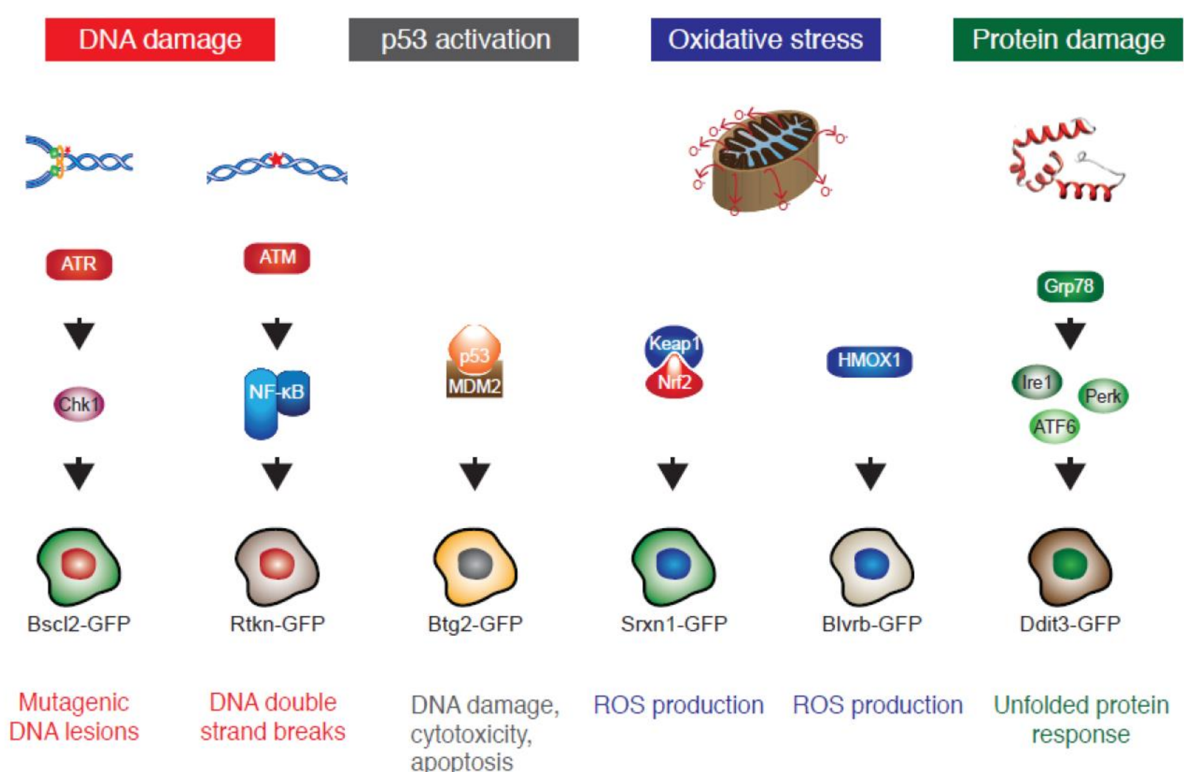

Figure 2: Biological endpoints detected by ToxTracker assay

Test Procedure
Cytotoxicity testing/dose range finding.
For chemical testing, first a dose range finding was performed using wild-type mES cells (strain B4418). Wild-type mES cells were exposed to 20 different concentrations of the test

substances, with a maximum concentration of 10 mM. Cytotoxicity was estimated by cell count after 24 h exposure using a flow cytometer and was expressed as the percentage of viable cells after 24 h exposure compared to vehicle control exposed cells. From this dose range finding, 5 concentrations were selected.

The six independent mES reporter cell lines were seeded in gelatin-coated 96-well cell culture plates in 200 µl mES cell medium (50000 cells per well). 24 h after seeding the cells in the 96-well plates, medium was aspirated and fresh mES cell medium containing 10% fetal calf serum and the diluted chemicals were added to the cells. For the tested materials, five concentrations were tested in 2-fold dilutions. Induction of the GFP reporters was determined after 24 h exposure using a flow cytometer. Only GFP expression in intact single cells was determined. Mean GFP fluorescence was measured and used to calculate GFP reporter induction compared to a vehicle control treatment.

Cytotoxicity was estimated by cell count after 24 h exposure using a flow cytometer and was expressed as percentage of intact cells after 24 h exposure compared to vehicle exposed controls. For cytotoxicity assessment in the ToxTracker assay, the relative cell survival for the six different reporter cell lines was averaged, because the cytotoxicity levels were very similar. Metabolic activation was included in the ToxTracker assay by addition of S9 liver extract from aroclor 1254- induced rats (Moltox). Cells were exposed to five concentrations of the test samples in the presence of 0.25% S9 and required co-factors (RegenSysA+B, Moltox) for 24 h.
In case auto-fluorescence of the test substances was observed in the dose range finding, wild-type mES cells were exposed to the test samples at the same concentrations as used in the ToxTracker assay. The mean fluorescence caused by the sample was then subtracted from the ToxTracker results of the sample.

Positive reference treatments with cisplatin (DNA damage), diethyl maleate (oxidative stress), tunicamycin (unfolded protein response) and aflatoxin B1 (metabolic activation of progenotoxins by S9) were included in all experiments.

Criteria
The ToxTracker assay was considered to have a positive response when a compound induces at least a 2-fold increase in GFP expression in any of the reporters. Activation of the Bscl2-GFP or Rtkn-GFP reporters indicate induction of DNA damage, Srxn1-GFP and Blvrb-GFP indicate induction of cellular oxidative stress and Ddit3-GFP activation is associated with the unfolded protein response. The Btg2-GFP reporter is controlled by the p53 tumour suppressor and is activated by DNA damage but can also be induced by oxidative stress, hypoxia, metabolic stress and apoptosis.

Results on Hydroxypropyl p-phenylenediamine

- Limited cytotoxicity (~25%) was observed at the maximum tested concentration of 1250 µM in the absence of a metabolising system. A small decrease in cytotoxicity was observed in the presence of a metabolising system. The maximum test concentration was limited due to precipitation in the cell culture medium after 24 h of treatment.
- Weak (>1.5-fold) activation of the Bscl2-GFP genotoxicity reporter was observed in absence of a metabolising system, but the 2-fold threshold for a positive ToxTracker was not reached.
- Activation of the p53 response in absence of a metabolising system was observed.
- Activation of the oxidative stress reporters was observed in the ToxTracker assay in the absence and presence of a metabolising system.
- Activation of the unfolded protein response was observed in absence and presence of S9.

Conclusion
Under the conditions of this study, hydroxypropyl p-phenylenediamine was classified as non-genotoxic in the ToxTracker assay since no activation of more than 2-fold of the Bscl2-GFP or Rtkn-GFP reporter was observed. Hydroxypropyl p-phenylenediamine activated the p53 response, oxidative stress and proteins stress reporters.

Derr and Brandsma, 2021

SCCS comment
ToxTracker assay is validated but has not been adopted yet as an OECD TG. The Applicant did not present raw data, which are needed by the SCCS for a proper assessment of the results.
The SCCS acknowledges that reporter gene assays based on human, animal or bacterial cells are tools supporting a WoE approach. The results of the Toxtracker will be used as such by the SCCS.

Hydroxypropyl p-phenylenediamine HCl

***In Vitro* Alkaline Comet Assay in Reconstructed 3D Full Thickness Human Skin Models**

Test Material
Hydroxypropyl p-phenylenediamine HCl batch E64 (purity 99.8%) was used in this study.

Test System
The Phenion® full-thickness skin model used in this assay.

Test Procedure
The Phenion® full thickness skin model consisted of human primary keratinocytes and fibroblasts from single donor origin and was obtained from Henkel (Germany). Tissue has well developed epidermis and dermis layer and fully functional barrier layer that mimic the native human skin.

Two valid main experiments were performed. Prior to the main experiments, a dose range finding study was performed to select suitable dose levels for the comet assay.
Tissues were treated for three times (0, 24, and 45 hours ± 30 min). In the confirmatory trial of the Comet assay, aphidicolin (APC) was added to the culture 4 hours ± 15 minutes prior to harvest.
In the preliminary cytotoxicity test for selection of dose levels, tissues were exposed to vehicle alone (ethanol in water 70:30) and to 11 concentrations of test article with half dose spacing. One tissue was used per concentration. The test article was evaluated at a maximum concentration of 1600 µg/cm².
In the definitive Comet assay, the concentrations of 1, 2, 3, 8, 15, 25 and 50 µg/cm² of test article were tested using triplicate tissues based on the cytotoxicity profile.
A first confirmatory assay, where the positive control for the dermis did not meet assay criteria, and a repeat confirmatory assay were conducted at concentrations of 1.25, 2.5, 5, 10, 15, 20, 25, and 50 µg/cm².
The highest dose evaluated for comets following a 3-day dosing regimen was selected based on visible precipitate observed on top of the tissue at the end of the last treatment.
Negative (Ethanol in water 70:30) and positive controls (methyl methane sulfonate MMS and benzo[a]pyrene B[a]P in the definitive and confirmatory/repeat-confirmatory experiments, respectively) were run in parallel.
Test article dilutions were prepared immediately before use and delivered to the test system at room temperature under filtered light.

Cytotoxicity was determined in each experiment based on measurement of adenylate kinase (AK) activity and intracellular Adenosine Triphosphate (ATP) concentration. Coded duplicate

comet assay slides per skin membrane were evaluated (50 cells per slide) for both epidermal and dermal cells using the fluorescent dye SYBR Gold to determine the genotoxic potential.

The following endpoints of DNA damage were assessed and measured:
• Comet Tail Migration: defined as the distance from the perimeter of the Comet head to the last visible point in the tail.
• % Tail DNA (also known as % tail intensity or % DNA in tail): defined as the percentage of DNA present in the tail.
• Tail Moment: defined as the product of the amount of DNA in the tail and the tail length [(% Tail DNA x Tail Length)/100]

The study was conducted under GLP.

Results
In the definitive Comet assay, post-treatment cytotoxicity [≥ 2-fold increase in adenylate kinase activity or ≥ 50% reduction in ATP/protein ratio relative to the vehicle control] was not observed at any concentration. At the end of the third treatment, visible precipitate was observed on top of the tissue at concentrations ≥ 15 μg/cm².

Hydroxypropyl p-phenylenediamine HCl gave a negative (non-DNA damaging) response in this assay in the dermis and epidermis of the Reconstructed 3D Human Skin Model. None of the test article-treated multi-well slides showed significant increases in the % Tail DNA compared to the vehicle control in the epidermis. Test article treated multi-well slides at 2 μg/cm² showed a significant decrease in the % Tail DNA compared to the vehicle control in the dermis. However, this decrease was within historical control limits and not dose-related. Therefore, this decrease was not considered biologically relevant. The arithmetic mean of the solvent control did not exceed 20% tail intensity in either the dermis and epidermis, and the positive control induced at least a 2-fold increase in % tail intensity and an absolute difference of at least 15% (MMS) compared to the vehicle control in both the dermis and epidermis.

Since the result of the definitive Comet assay was negative, a confirmatory Comet assay was conducted. In the initial confirmatory Comet assay, post-treatment cytotoxicity was not observed at any concentration. At the end of the third treatment, visible precipitate was observed on top of the tissue at concentrations ≥ 10 μg/cm².

The test article gave a negative (non-DNA damaging) response in this assay in the epidermis of the Reconstructed 3D Human Skin Model. However, the positive control for the dermis did not meet assay criteria. Therefore, the confirmatory assay was repeated with the same concentrations as in the initial confirmatory assay.
In the repeat confirmatory Comet assay, post-treatment cytotoxicity was not observed at any concentration. At the end of the third treatment, visible precipitate was observed on top of the tissue at concentrations ≥ 15 μg/cm².

Hydroxypropyl p-phenylenediamine HCl gave a negative (non-DNA damaging) response in this assay in the dermis and epidermis of the Reconstructed 3D Human Skin Model. None of the test article-treated multi-well slides showed significant increases in the % Tail DNA compared to the vehicle control in the dermis and epidermis. The arithmetic mean of the solvent control did not exceed 20% tail intensity in either the dermis and epidermis, and the positive control induced at least a 2-fold increase in % tail intensity and an absolute difference of at least 5% (B[a]P + APC) compared to the vehicle control in both the dermis and epidermis.

Table 13. Summary of the definitive comet assay results in 3D human skin model.

Treatment (µg/cm²)		Cytotoxicity		Dermis Tail Intensity							Epidermis Tail Intensity						
		AK (Fold)	ATP/ Protein (%)	Cell#/ Tissue	Tissue 1	Tissue 2	Tissue 3	Mean	±	SD	Cell#/ Tissue	Tissue 1	Tissue 2	Tissue 3	Mean	±	SD
70% Ethanol	0.0	1.0	0.0	150	5.45	8.27	6.66	6.79	±	1.41	150	4.28	2.10	1.36	2.58	±	1.52
R0025521B	2.0	0.8	0.0	150	3.31	3.53	2.61	3.15	±	0.48**	150	0.49	7.12	6.50	4.70	±	3.66
	8.0	0.6	0.0	150	2.57	6.38	4.26	4.40	±	1.91	150	2.11	0.53	2.21	1.62	±	0.94
	15.0	0.7	0.0	150	4.59	4.92	4.08	4.53	±	0.42	150	5.44	3.45	1.97	3.62	±	1.74
MMS	5.0	0.2	5.3	150	30.37	25.71	30.93	29.00	±	2.87**	150	36.09	29.99	34.05	33.38	±	3.11**

*p≤0.05, **p≤0.01, As compared to vehicle control (Dunnett's test or T-Test)
AK = Adenylate Kinase Activity
ATP = Adenosine Triphosphate
MMS = Methyl methanesulfonate
SD = Standard Deviation

Conclusion
Under the conditions of the study, hydroxypropyl p-phenylenediamine HCl did not cause a significant increase in DNA damage in the dermis and epidermis of Reconstructed 3D Human Skin Models relative to the concurrent vehicle control. Therefore, hydroxypropyl p-phenylenediamine HCl was concluded to be negative in the *in vitro* 3D Skin Comet Assay.

Roy, 2021

SCCS comment
The *in vitro* comet assay on 3D-skin model is validated but has not been adopted yet as OECD TG. The SCCS acknowledges that the comet assay is a tool supporting a WoE approach. The results of this test will be used as such by the SCCS.

TOXTRACKER

Test Materials
Hydroxypropyl p-phenylenediamine HCl batch E64 was used in this study.

Test System and procedure
The ToxTracker assay for evaluation of genotoxicity of Hydroxypropyl p-phenylenediamine HCl was conducted together with evaluation of Hydroxypropyl p-phenylenediamine. Details of procedure are described above.

Results
Hydroxypropyl p-phenylenediamine 2HCl
- Significant cytotoxicity (>50%) was observed at a concentration of 1891 µM in the absence of a metabolising system. A small decrease in cytotoxicity was observed in the presence of a metabolising system.
- No activation of the Bscl2-GFP or Rtkn-GFP genotoxicity reporters was observed in absence or presence of a metabolising system.
- Activation of the p53 response in absence of a metabolising system was observed.
- Activation of the oxidative stress reporters was observed in the ToxTracker assay in the absence and presence of a metabolising system.
- Activation of the unfolded protein response was observed in presence of S9.

Conclusion
Under the conditions of this study, hydroxypropyl p-phenylenediamine was classified as non-genotoxic in the ToxTracker assay since no activation of more than 2-fold of the Bscl2-GFP or Rtkn-GFP reporter was observed. Hydroxypropyl p-phenylenediamine activated the p53 response, oxidative stress and proteins stress reporters.

Derr and Brandsma, 2021

SCCS comment
ToxTracker assay is validated but has not been adopted yet as an OECD TG. The Applicant did not present raw data, which are needed by the SCCS for a proper assessment of the results.
The SCCS acknowledges that reporter gene assays based on human, animal or bacterial cells are tools supporting a WoE approach. The results of the Toxtracker will be used as such by the SCCS.

In Vitro Mammalian Cell Gene Mutation Test of hydroxypropyl p-phenylenediamine 2HCl

Guideline:	OECD TG 476, Commission Regulation (EC) No. 440/2008
Cells:	Chinese Hamster Ovary Cells CHO K1 cells [*Hprt* locus for 6-thioguanine (6-TG) resistance]
Replicates:	Duplicate cultures in two independent experiments
Test substance:	Hydroxypropyl p-phenylenediamine HCl (salt)
Batch:	R0025521B E64
Purity:	Relative purity 99.8%
Solvent:	Purified water diluted 10-fold in treatment medium
Metabolic activation:	Phenobarbital (PB) and β-naphthoflavone (BNF) induced rat liver
Positive controls:	Ethyl methanesulfonate (EMS), 7,12-Dimethylbenz[a]anthracene (DMBA)
Concentrations:	In the presence of S9-mix: 2000, 1800, 1600, 1500, 1400, 1200, 1000, 500, 250 and 125 µg/mL In the absence of S9-mix: 2000, 1000, 800, 700, 600, 500, 400, 200, 100 and 50 µg/mL
Treatment:	5h without and with S9-mix;
Expression period:	7 days
GLP:	In compliance
Study period:	March 14 – July 13, 2023

An *in vitro* mammalian cell assay was performed in CHO K1 Chinese hamster ovary cells at the *Hprt* locus to evaluate the potential of R0025521B to cause gene mutation. Treatments were carried out for 5 hours with and without metabolic activation (±S9-mix). The design of this study was based on the Commission Regulation (EC) No. 440/2008 and OECD No. 476 guideline, and the study was performed in compliance with Charles River Laboratories Hungary Kft. standard operating procedures and with the OECD Principles of Good Laboratory Practice.
Distilled water was used as the vehicle (solvent) of the test item in this study. Treatment concentrations for the mutation assays of the main tests were selected based on the results of a preliminary toxicity test as follows:

Assay 1 repeated
5-hour treatment in the presence of S9-mix:
2000, 1800, 1600, 1500, 1400, 1200, 1000, 500, 250 and 125 µg/mL.

Assay 1
5-hour treatment in the absence of S9-mix:
2000, 1000, 800, 700, 600, 500, 400, 200, 100 and 50 µg/mL.

Assay 2
5-hour treatment in the presence of S9-mix:
2000, 1800, 1600, 1500, 1400, 1200, 1000, 500, 250 and 125 µg/mL.

5-hour treatment in the absence of S9-mix:
2000, 1000, 800, 700, 600, 500, 400, 200, 100 and 50 μg/mL.

In Assay 1 (repeated) insolubility was detected in the final treatment medium at the end of the treatment in the experiments with metabolic activation at the concentration range of 2000 – 1200 μg/mL. There were no large changes in pH and osmolality after treatment in any cases.

In Assay 1 (repeated), in the presence of S9-mix (5-hour treatment), cytotoxicity of the test item was observed (1600, 1500 and 1400 μg/mL concentrations showed, with a relative survival of 12%, 44% and 68%, respectively). An evaluation was made using data of eight of the lower concentrations based on the acceptable cytotoxicity data. Statistically significant increase in the mutation frequencies were observed in this experiment at the concentrations of 1500 μg/mL, 1400 μg/mL and 500 μg/mL at $p < 0.01$ level; and at the concentrations of 1200 μg/mL and 125 μg/mL at $p < 0.05$ level, although the observed values were within the local, general historical control range. Furthermore, the observed mutant frequencies (11.6, 8.4, 8.8, 10.5 and 8.9 x 10^{-6}) were within the expected range of the negative control samples according to the relevant OECD guideline (expected range: 5-20 x 10^{-6}). No dose response to the treatment was observed (a trend analysis showed no effect of treatment (R^2 = 0.045)). In this assay, precipitation was observed from 1200-1400 μg/mL, so in theory any higher concentrations can be excluded according to the guideline; however, the interpretation for this assay is identical. This experiment is considered to be negative.

In Assay 1, in the absence of S9-mix (5-hour treatment), cytotoxicity of the test item was observed 800, 700, 600 and 500 μg/mL concentrations showed, with a relative survival of 12%, 23%, 30% and 42%, respectively). An evaluation was made using data of eight of the lower concentrations based on the acceptable cytotoxicity data. A statistically significant increase in the mutation frequencies was observed in this experiment at the concentration of 500 μg/mL (at $p < 0.05$ level), although the observed values were within the general historical control range. Furthermore, the observed mutant frequencies (8.2 x 10^{-6}) were within the expected range of the negative control samples according to the relevant OECD guideline (expected range: 5-20 x 10^{-6}). A slight dose response was indicated by the linear trend analysis because all concentrations were within the expected range, it was not considered to be relevant (R^2 = 0.890). This experiment is considered to be negative.

In Assay 2, a 5-hour treatment with metabolic activation (in the presence of S9-mix) and a 5-hour treatment without metabolic activation (in the absence of S9-mix) were performed.
For the 5-hour treatment in the presence of S9-mix, the following concentrations were examined: 2000, 1800, 1600, 1500, 1400, 1200, 1000, 500, 250 and 125 μg/mL.
For the 5-hour treatment in the absence of S9-mix, the following concentrations were examined: 2000, 1000, 800, 700, 600, 500, 400, 200, 100 and 50 μg/mL.
In Assay 2, insolubility (precipitation) was detected in the final treatment medium at the end of the treatment in the experiment with metabolic activation at the concentration range of 2000 – 1400 μg/mL. Also, an indication of possible insolubility was seen at 500 – 250 μg/mL as an oily film; but since higher concentrations showed no evidence of insolubility, these observations were not considered to be relevant to study interpretation. There were no large changes in pH and osmolality after treatment in any cases.

In the presence of S9-mix (5-hour treatment), cytotoxicity of the test item was observed (1600, 1500 and 1400 μg/mL concentrations showed, with a relative survival of 9%, 28% and 64%, respectively). An evaluation was made using data of eight of the lower concentrations based on the acceptable cytotoxicity data. A statistically significant increase in the mutation frequencies (at $p < 0.05$ level) was observed in this experiment at the concentration of 1000 μg/mL, although the observed values were within the local, general historical control range. Furthermore, the observed mutant frequency (12.8 x 10^{-6}) was within the expected range of the negative control samples according to the relevant OECD guideline (expected range: 5-20 x 10^{-6}). No dose response to the treatment was observed (a trend

analysis showed no effect of treatment (R^2 = 0.037)). In this assay, precipitation was observed from 1600 µg/mL, so in theory any higher concentrations can be excluded according to the guideline; however, the interpretation for this assay is identical. This experiment is considered to be negative. Moreover, it confirmed the result of the Assay 1.

In the absence of S9-mix (5-hour treatment), cytotoxicity of the test item was observed (800, 700, 600, 500 and 400 µg/mL concentrations showed, with a relative survival of 8%, 18%, 23%, 39% and 57%, respectively). An evaluation was made using data of eight of the lower concentrations based on the acceptable cytotoxicity data. A statistically significant increase in the mutant frequencies (at $p < 0.01$ level) was observed in this experiment at the concentration of 800 µg/mL and 400 µg/mL, although the observed values were within the general historical control range. Furthermore, the observed mutant frequency (14.1 and 18.5 x 10^{-6}) was within the expected range of the negative control samples according to the relevant OECD guideline (expected range: 5- 20 x 10^{-6}). No dose response to the treatment was observed (a trend analysis showed no effect of treatment (R^2 = 0.431)). This experiment is considered to be negative.

The spontaneous mutant frequency of the negative (vehicle) control was in accordance with the general historical control range in all assays. The positive controls gave the anticipated increases in mutant frequency over the controls and were very much in line with the historical data in all assays. Eight evaluated concentrations were presented in all assays. The cloning efficiencies for the negative controls at the beginning and end of the expression period were within the target range. The evaluated concentration ranges were considered to be adequate (concentrations were tested up to the maximum recommended concentrations or cytotoxic range in each test). The overall study was considered to be valid.

In conclusion, no mutagenic effect of R0025521B was observed either in the presence or absence of a metabolic activation system under the conditions of this HPRT assay. The study was considered valid based on the negative and positive control values.

Nagy, 2023

SCCS comments

The SCCS agrees that hydroxypropyl p-phenylenediamine 2HCl is negative in the mammalian gene mutation test. The SCCS has following comments:

- In the presence of S9 mix a significant increase in mutant frequency was detected in 5 concentrations in assay 1 and in 1 concentration in assay 2. However, most of values were in the range of historical control or very slightly above but within recommended level of spontaneous mutant frequency (5-20x10^6). No concentration response was found.
- In the absence of S9 mix significant increase in mutant frequency was observed in one concentration in both assays which was above the range of historical control (18.5x10^6 vs 4.7x10^6 in assay 2) but within recommended level of spontaneous mutant frequency (5-20x10^6). No concentration response was found.
- Precipitation (insolubility) occurred in concentration 500µg/ml and above in the absence of metabolic activation and in concentrations 1600µg/ml and above in the presence of S9mix.
- High variability in negative and positive historical control values (high standard deviation, difference in min and max) was observed (in spontaneous mutant frequency almost 20x difference), for positive controls 5-15x difference).

Summary of all results and overall SCCS comment on mutagenicity/genotoxicity of Hydroxypropyl p-phenylenediamine base and salt:

Table 14. Summary of all results from submission I and II:

Test method	Hydroxypropyl p-phenylenediamine (base)	Hydroxypropyl p-phenylenediamine 2HCl
In vitro		
Gene mutation in bacteria (Ames test)	Negative	Positive
Mammalian cell gene mutation tests	Not tested	Inconclusive
Micronucleus test	Positive	Positive
3D micronucleus test*	Negative	Not tested
3D Comet assay*	Not tested	Negative
ToxTracker*	Negative	Negative
In vivo		
Mammalian micronucleus test	Not tested	Negative
New mammalian gene mutation test	Not tested	Negative

**New tests available in submission II*

New data

New data

The Applicant used a battery of assays to test genotoxicity of Hydroxypropyl p-phenylenediamine base and its salt according to the SCCS Guidance for the testing of cosmetic ingredients and their safety evaluation (SCCS 1647/22) and the current state-of-knowledge.

Hydroxypropyl p-phenylenediamine base was negative in the bacterial gene mutation test but positive in a standard micronucleus assay (from the SCCS/1608/19 Opinion Submission I). As follow up Micronucleus assay was performed on the Reconstructed skin. RSMN assay is a promising test to be used as second tier assay to follow-up on positive results from standard *in vitro* assays for dermally applied compounds even if RSMN is not adopted yet as an OECD TG. The SCCS agrees that hydroxypropyl p-phenylenediamine base is negative for the induction of micronuclei in this assay. Additionally negative results on ToxTracker reporter gene assay based on stem cells support this finding in WoE approach.

Hydroxypropyl p-phenylenediamine HCl was positive in micronucleus *in vitro* but negative on *in vivo* micronucleus (previous opinion SCCS/1608/19) demonstrating no *in vivo* chromosomal damage. Hydroxypropyl p-phenylenediamine HCl was also negative in a new mammalian gene mutation test under experimental conditions tested. Additionally, the comet assay results on reconstructed skin model and results on stem cells-based reporter genes assay (ToxTracker) were negative in the experimental conditions tested.
The SCCS is of the opinion that Hydroxypropyl p-phenylenediamine base and its salt do not induce gene mutations in mammalian cells nor structural or numerical chromosomal damage in experimental conditions tested up to precipitation concentrations.

3.4.6.2 Mutagenicity / genotoxicity *in vivo*

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3.4.7 Carcinogenicity

From previous Opinion (SCCS/1608/19)

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.

SCCS comment
After analysis of the genotoxicity and other data, the SCCS considers that A165 is not likely to be a carcinogen.

3.4.8 Photo-induced toxicity

3.4.8.1 Phototoxicity / photo-irritation and photosensitisation

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3.4.8.2 Photomutagenicity / photoclastogenicity

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3.4.9 Human data

/

3.4.10 Special investigations

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3.5 SAFETY EVALUATION (INCLUDING CALCULATION OF MoS)

NOAEL (30 mg/kg/day), identified in a study for effects of hydroxypropyl p-phenylenediamine 2HCl on embryo-foetal development and in a 90-day repeated oral toxicity study, was chosen for MOS calculation.

The dermal absorption of hydroxypropyl p-phenylenediamine 2HCl was estimated to be 3.7 μg/cm2 (mean + 1SD).

Absorption through the skin	**DA_a**	**=**	**3.7 μg/cm²**
Skin Surface area	**SSA**	**=**	**580 cm²**
Dermal Absorption per treatment	**SSA x DA_a x 0.001***	**=**	**2.146 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SSA x DA x 0.001* x f**/bw**		
		=	**0.036 mg/kg bw**
No observed adverse effect level	**NOAEL**	**=**	**30 mg/kg bw/d**
(90 day tox study and developmental tox study, rat)			
Bioavailability 50%* **		**=**	**15 mg/kg bw/d**
Margin of Safety	**adjusted NOAEL/SED**	**=**	**417**

* conversion factor μg to mg
** f = frequency of application (1 in this case)
*** adjusted NOAEL considering 50% bioavailability

3.6 DISCUSSION

The ingredients hydroxypropyl p-phenylenediamine and its dihydrochloride salt (A165) are intended to be used in oxidative hair colouring products at on-head concentrations of up to 2%. The safety profile of hydroxypropyl p-phenylenediamine and its dihydrochloride salt has been evaluated by SCCS in 2019 (Opinion SCCS/1608/19). The SCCS concluded that hydroxypropyl p-phenylenediamine and its dihydrochloride salt are not safe when used in oxidative hair colouring products due to potential genotoxicity.

Complementary tests on both forms of A165 were performed in order to fulfill SCCS requests and further explore its genotoxicity profile. Accordingly, the following tests were conducted namely:
For hydroxypropyl p-phenylenediamine base:

- the reconstructed skin micronucleus assay (RSMN)
- ToxTracker – to investigate the genotoxic mode-of-action

For hydroxypropyl p-phenylenediamine dihydrochloride form:

- the mammalian gene mutation test on CHO K1 Chinese hamster ovary cells at the *Hprt* locus,
- *in vitro* alkaline Comet assay in reconstructed 3D full thickness human skin model with
- ToxTracker to investigate the genotoxic mode-of-action

Additionally new analytical data have been provided.

Physicochemical properties

Impurities at content above 0.1% have been quantified using either external reference standards of the corresponding impurities or area normalisation. In the 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. It should be demonstrated that the main compound and all the impurities are fully dissolved in the dilution solvent prior to the HPLC analysis. All impurities above 0.1% should be accurately quantified in every batch; effort should be made during the manufacturing procedure to keep these impurities at trace levels.

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 that were 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 is not 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.

Dermal absorption
The dermal absorption of hydroxypropyl p-phenylenediamine 2HCl was estimated to be 3.7 µg/cm2 (mean + 1SD).

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

Hydroxypropyl p-phenylenediamine (base form) was non mutagenic in a bacterial reverse mutation test. It was positive in an *in vitro* micronucleus test in cultured human lymphocytes. This result can be considered not biologically relevant as it was not confirmed in a second-tier *in vitro* micronucleus test using EpiDermTM human reconstructed skin. In WoE hydroxypropyl p-phenylenediamine was investigated for the genotoxic mode-of-action using reporter genes Toxtracker assay with negative results. Thus, the SCCS considers hydroxypropyl p-phenylenediamine (base form) neither mutagenic nor genotoxic.

Hydroxypropyl p-phenylenediamine dihydrochloride salt was mutagenic in the bacterial reverse mutation test. *In vitro* mammalian gene mutation tests were considered not reliable due to high variability between experiments. To further evaluate a mutagenic potential, new mammalian gene mutation test on CHO K1 Chinese hamster ovary cells at the *Hprt* locus was provided with negative results.
Hydroxypropyl p-phenylenediamine 2HCl was also negative in an *in vitro* alkaline Comet assay in the reconstructed 3D full thickness human skin model.

Hydroxypropyl p-phenylenediamine 2HCl was positive in an *in vitro* micronucleus test. However, it 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 bw.

Furthermore, the negative outcome of hydroxypropyl p-phenylenediamine dihydrochloride salt in ToxTracker can be used in a WoE approach to support the finding that the salt form of A165 is neither mutagenic nor genotoxic.

Based on the data provided, the SCCS considers both the base and the salt form of hydroxypropyl p-phenylenediamine to have no mutagenic/genotoxic potential.

4 CONCLUSION

The SCCS concludes the following:

1. *In light of the data provided, does the SCCS consider Hydroxypropyl p-phenylenediamine and its dihydrochloride salt 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 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 in cosmetic products?*

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

5 MINORITY OPINION

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6 REFERENCES

Bonnet B (2022). *Hydroxypropyl p-phenylene diamine and its dihydrochloride salt* A165 H p-phenylenediamine; Analytical file. May 6, 2022

Curren RD., Mun GC., Gibson DP., Aardema MJ (2006). Development of a method for assessing micronucleus induction in a 3D Human skin model (EpiDermTM), Mutation Res. 607: 192–204.

Derr R and Brandsma I (2021). ToxTracker Test report. Toxys Study No. 20069. 6 April 2021.

Imexine OBN: Determination of the Partition Coefficient (n-Octanol/Water) by High Performance Liquid Chromatography (HPLC). IBACON Study No. 131461186, 2019.

Nagy I (2023). An *In Vitro* Mammalian Cell Gene Mutation Test of R0025521B. Test Facility Study Code: 23-064-015C. 14 July 2023.

Roy S (2020). R0025521A: In Vitro Micronucleus Test using Reconstructed skin Micronucleus (RSMN) assay in EpiDermTM. Bioreliance Study No. AF89DK.358.BTL.

Roy S (2021). R0025521B: In Vitro Alkaline Comet Assay in Reconstructed 3D Full Thickness Human Skin Models. Bioreliance Study No. AF84JB.359R.BTL.
SCCS, 2019. Opinion on Hydroxypropyl p-phenylenediamine and its dihydrochloride salt (A165). SCCS/1608/19. 30-31 October 2019
https://ec.europa.eu/health/sites/default/files/scientific_committees/consumer_safety/docs/sccs_o_230.pdf

SCCS, 2023. The SCCS notes of guidance for the testing of cosmetic ingredients and their safety evaluation. 12th revision. SCCS/1647/22.. 16 May 2023. SCCS Notes of guidance for the testing of cosmetic ingredients and their safety evaluation - 12th revision (europa.eu)

Toner F (2014). The In Vitro Percutaneous Absorption of Radiolabelled R0025521B Through Human Skin. Study No. 795069, Report No. 35281.
https://pubchem.ncbi.nlm.nih.gov/compound/15907303#section=Names-and-Identifiers
http://www.abichem.com/pro_result/?id=2518565

From previous opinion (SCCS/1608/19)

Amsellem C. (2014). Primary Cutaneous Tolerance. Prediction of the irritant potential on human reconstructed epidermis EpiskinSM model- D13. Episkin Study No. 14-BPL-0028.

Analytical File of hydroxypropyl p-phenylenediamine and its hydrochloride salt (2014)

Gohel D (2014a). Repeated Dose 90-Day Oral Toxicity Study of R0025521B In Wistar Rats. JRF Study No. 443-1-03-5651. Volume I

Gohel D (2014b). Repeated Dose 90-Day Oral Toxicity Study of R0025521B In Wistar Rats. JRF Study No. 443-1-03-5651. Volume II

Hall et al. (2012). Liver Hypertrophy: A Review of Adaptive (Adverse and Non-adverse) Changes—Conclusions from the 3rd International ESTP Expert Workshop. Toxicologic Pathology, 40: 971-994

Hobson S (2012). R0025521B: Reverse Mutation in five histidine-requiring strains of Salmonella typhimurium. Covance Study No. 8268086.

Lloyd M (2013). R0025521B: Mutation at the hprt locus of Mouse Lymphoma L5178Y Cells (MLA) using the Microtitre® Fluctuation Technique. Covance Study No. 8268091.

Maillet S (2012). Ocular primary irritation B.C.O.P. – Study Performed on the Isolated Bovine Cornea. Measurement of the Bovine Corneal Opacity and Permeability. IEC Report No 120727RD.

Patel M (2014). Prenatal Developmental Oral Toxicity Study of R0025521B In Wistar Rats. JRF Study No. 461-1-04-5652

7 GLOSSARY OF TERMS

See SCCS/1647/22, 12th Revision of the SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation – Appendix 15 - from page 158

8 LIST OF ABBREVIATIONS

See SCCS/1647/22, 12th Revision of the SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation – Appendix 15 - from page 158