Scientific Committee on Consumer Safety

SCCS

OPINION ON

Hair dye Methylimidazoliumpropyl p-phenylenediamine HCl (A166) (CAS 220158-86-1) Submission I

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

ACKNOWLEDGMENTS

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All Declarations of Working Group members are available on the following webpage:
http://ec.europa.eu/health/scientific_committees/experts/declarations/sccs_en.htm

This Opinion has been subject to a commenting period of a minimum eight weeks after its initial publication (from 20 August 2019 until 21 October 2019).
Comments received during this time period were considered by the SCCS. For this Opinion, comments received resulted in the following main changes: sections 3.1.5, 3.3.13, tables in toxicological evaluation section, and conclusion.

1. ABSTRACT

The SCCS concludes the following:

1. *In light of the data provided, does the SCCS consider Methylimidazoliumpropyl p-phenylenediamine HCl (A166), safe when used in oxidative hair colouring products up to a maximum on-head concentration of 2%?*

   Based on the full set of information provided by Applicant (including added information provided by Applicant during the commenting period), the SCCS considers Methylimidazoliumpropyl p-phenylenediamine HCl (A166), 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 Methylimidazoliumpropyl p-phenylenediamine HCl (A166) in cosmetic products?*

   The SCCS has noted that Methylimidazoliumpropyl p-phenylenediamine HCl (A166) is a strong skin sensitiser.

Keywords: SCCS, scientific opinion, hair dye, Methylimidazoliumpropyl p-phenylenediamine HCl (A166), Regulation 1223/2009, CAS 220158-86-1.

Opinion to be cited as: SCCS (Scientific Committee on Consumer Safety), Opinion on hair dye Methylimidazoliumpropyl p-phenylenediamine HCl (A166), preliminary version adopted on 29 July 2019, final version adopted on 30-31 October 2019, SCCS/1609/19.

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

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ISSN ISBN

Doi: ND-

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2. MANDATE FROM THE EUROPEAN COMMISSION

Background

Submission I on the hair dye Methylimidazoliumpropyl p-phenylenediamine HCl (A166) (CAS 220158-86-1) with the chemical name 1H-Imidazolium,3-[3-[(4-aminophenyl)amino]propyl]-1-methyl-,chloride, hydrochloride was transmitted by Cosmetics Europe in 2017.

The ingredient Methylimidazoliumpropyl p-phenylenediamine HCl (A166) is 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 Methylimidazoliumpropyl p-phenylenediamine HCl (A166), 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 Methylimidazoliumpropyl p-phenylenediamine HCl (A166) in cosmetic products?

3. OPINION

3.1 CHEMICAL AND PHYSICAL SPECIFICATIONS

3.1.1 Chemical identity

3.1.1.1 Primary name and/or INCI name

Methylimidazoliumpropyl p-phenylenediamine HCl (INCI)

SCCS comment
The salt used is chloride, dihydrochloride therefore the following name will be used throughout the whole Opinion:
Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl

3.1.1.2 Chemical names

1H-Imidazolium, 3-[3-[(4-aminophenyl)amino]propyl]-1-methyl-,chloride, hydrochloride (1:1:2)

1H-Imidazolium, 1-[3-[(4-aminophenyl)amino]propyl]-3-methyl-, chloride, dihydrochloride

1-(3-((4-Aminophenyl)amino)propyl)-3-methyl-1H-imidazol-3-ium chloride hydrochloride

Ref: Wohr 2019

3.1.1.3 Trade names and abbreviations

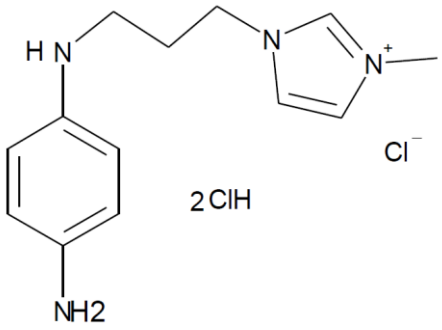
IMEXINE® OBL

Cosmetics Europe code A166
Other codes: R0027494A
C16778

3.1.1.4 CAS / EC number

CAS: 220158-86-1
EC: /

3.1.1.5 Structural formula



IR spectra, ^{1}H and ^{13}C NMR spectra of the studied batches were in accordance with the proposed structure.
Mass spectra of the studied batches were compatible with the proposed structure.
UV-Visible spectra of the studied batches were compatible with the proposed structure.

3.1.1.6 Empirical formula

$C_{13}H_{19}N_4$, Cl, 2HCl
or $C_{13}H_{21}N_4Cl_3$

Ref: Wohr 2019

3.1.2 Physical form

Very light beige to beige powder.

3.1.3 Molecular weight

Molecular weight: 339.70 g/mol.
Exact mass : 338.09

Note:
Cl: Molecular weight : 35.453
Cl: Exact mass : 34.97

3.1.4 Purity, composition and substance codes

The HPLC titre was carried out on all batches against batch R0027494A 019 L 001, reference standard considered as pure (99% w/w).
All batches have a titre ≥ 99% w/w.

The separation was achieved by reversed phase LC and performed on a HPLC system equipped with a photodiode array detector. Detection wavelength: λ = 245nm
All samples and eluents were filtered through a 0.2µm membrane filter (Pall Acrodisc GHP) prior to use.
The dilution solvent is a mixture of water + 0.01% of dithionite of sodium/acetonitrile 95/5[v/v].

Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) is the main component detected on the HPLC chromatograms: its HPLC purity is above 95% in all batches (Relative purity, UV- area %).
Some impurities are detected in the tested batches in particular in: Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) - batch 002 L 001.
This batch was one of the first synthetized batches.

According to the Applicant 'The impurities content in the other tested batches is very weak. The impurity content the future productions is now under control'.

One impurity was checked in all batches and detected:
(R0011102A: 4-Aminophenylamine dihydrochloride).
Its content was determined against reference standard R00011102A L 139, considered as pure (99.8% w/w):
R0011102A content: < 0.2% w/w in all batches.

Table 1: Comparative table of the main results (Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166)- batches : 009 D 003, 008 L 002, 002 L 001, 000 L 006)

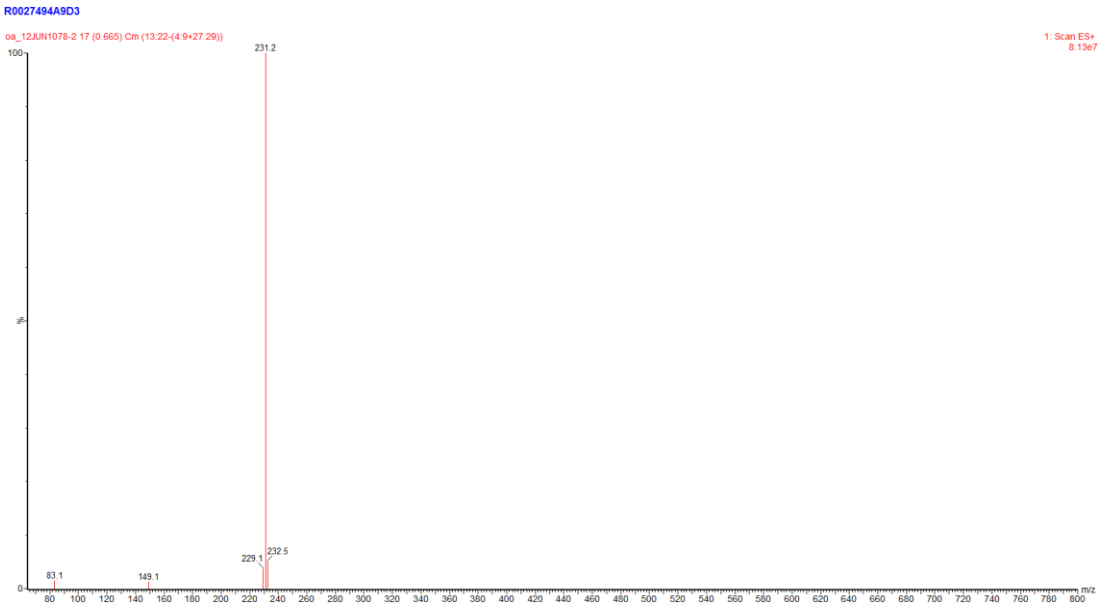
Analytical test	**009 D 003**	**008 L 002**	**002 L 001**	**000 L 006**
Identification				
Appearance	Light beige powder	Very light beige powder	Light beige powder	Beige powder
Infrared spectrometry	In accordance with the proposed structure			
UV spectrometry	Compatible with the proposed structure			
NMR spectrometry	In accordance with the proposed structure			
Mass spectrometry	Compatible with the proposed structure		Compatible with the proposed structure	
Assays				
HPLC Titre (1) (% w/w)	99.3 ± 0.3	99.0 ± 0.35	99.3 ± 0.6	99.5 ± 0.1
HPLC Profile (2) (UV purity- Area %)	Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) > 95 % Some impurities are detected			
R0011102A (3) (impurity content by HPLC): (µg/g)	328	633	638	1595
Water content *(for information)*	1.1% w/w *(KF method)*		0.8% w/w (calculated from elemental analysis)	
Chloride content Elemental analysis (Theoretical value: 31.3% w/w)	31.0	31.5	30.6	31.1

Note: in Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) *batch 002 L 001, 4 % of* Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) *is under sulphate form (calculated from corresponding elemental analysis).*

(1) *Titre against batch R0027494A 019 L 001 reference standard considered as pure (99.0% w/w)*
(2) *UV detection: UV purity - Area %, without response factor.*
Irrespective of residual solvents, salts and other non-detectable products
(3) *Determination of 4-aminophenylamine dihydrochloride against batch R0011102A 000 L 139 reference standard considered as pure (99.8% w/w)*

The main results of the four batches are comparable.

Mass spectra
According to the Applicant, an Analysis FIA / MS on Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) batch 009 D 003 was realized via OpenLynx.
The expected cation was mainly detected. Presence of secondary ions not characterised by low intensity.



Ref: Analytical file – Imexine OBL – June 2017

SSCS comment
The purity of the test substance has been quantified by HPLC against batch R0027494A 019 L 001, reference standard considered as pure (99.0% w/w).

3.1.5 Impurities / accompanying contaminants

Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) is the main component detected on the HPLC chromatograms: its HPLC purity is above 95% in all batches (Relative purity, UV- area %).

Some impurities are detected in the tested batches in particular in: Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) - batch 002 L 001. This

batch was one of the first synthesized batches. According to the Applicant, the impurity content in future productions is now under control.

The determination of R0011102A was carried out by HPLC (external calibration) in the four batches against a primary reference standard:

R0011102A batch 000 L 139 (purity considered at 99.8%).
R0011102A 000 L 139 is a dichlorhydrated form.

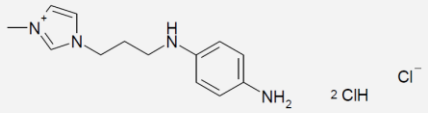
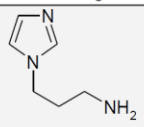
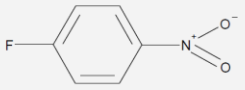
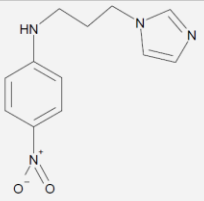
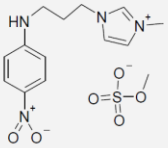
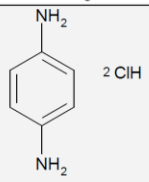
R0011102A content in Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166):
- Batch 009 D 003: 328 µg/g
- Batch 008 L 002: 633 µg/g
- Batch 002 L 001: 638 µg/g
- Batch 000 L 006: 1595 µg/g

R0011102A content: < 0.2% w/w in all batches.

For Batches 009 D 003 and 008 L 002, no impurity has been detected.
For Batch 002 L001, impurities eluting at 2.4, 7.8, and 8.8 min. have been detected, identified and quantified (area %) as 3-aminopropyl imidazole (0.1), R0027060A (0.2) and R0026760A (0.3) respectively. Impurities eluting at 6.4 min (< 0.1) and traces at 3.7min and 5.3 min (without identification) are detected.
For batch 000 L 006, only impurities eluting at 2.4 and 3.7 min have have been detected, identified and quantified (area %) as 3-aminopropyl imidazole (0.1) and R0011102A (0.2) respectively.

Impurities, which have a content ≥ 0.1% (area% without response factor – UV detection), were identified using a standard mixture.

Potential impurities in Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) can originate from starting material, synthesis intermediates, or by-products.

Imexine OBL (Main product)	 Molecular weight= 339.696
3-aminopropyl imidazole (Synthesis reagent)	 Molecular Weight =125.17
R0018727A (Starting material)	 Molecular Weight =141.10
R0026760A (N-2)	 Molecular Weight =246.27
R0027060A (N-1)	 Molecular weight = 372.41
R0011102A 4-aminophenylamine dihydrochloride (Potential impurity)	 Molecular weight =181.065

Residual solvents
Determination of residual solvents in Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) by Gas Chromatography (GC/HS - Standard addition method) (Results in µg/g).

Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166)	batch 009 D 003	batch 008 L 002	batch 002 L 001	batch 000 L 006
Chloroethane	< 500	< 25	< 500	# 560
Diethylether	< 100	< 100	< 100	< 200
Methylethylether	< 100	Not detected < 100	< 100	< 200
Ethanol	< 1000	< 500	< 1500	< 500
Chloropropane				< 500

Heavy metals content
Determination of metal content (µg/g unless otherwise specified : % w/w):
Dosage of 24 elements using ICP/AES, ICP-MS or atomic fluorescence (for Hg) after mineralization in microwave bomb according to method : CONS 285 LA2012
- Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) - Batch 009 D003 (µg/g except Na: % w/w):
  As, Cd, Hg, Pb, Pd, Sb : Each < 1
  Al, Ba, Co, Cr, Cu, Fe, Mn, Mo, Ni, Se, Sn, Ti, V, Zn : Each <5
  Ca, K, P: <50
  Na: 0.66 % (w/w)
- Methylimidazoliumpropyl p-phenylenediamine , Cl, 2HCl (A166)- Batch 008 L 002 (µg/g) :
  As, Cd, Hg, Pb, Pd, Sb : Each < 1
  Al, Ba, Co, Cr, Cu, Fe, Mn, Mo, Ni, Se, Sn, Ti, V, Zn : Each <5
  Ca, K, P, Na: <50
- Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) - Batch 002 L 001 (µg/g except Na: % w/w) :
  As, Cd, Hg, Sb : Each < 1
  Ba, Co, Cr, Cu, Mn, Mo, Ni, Se, Sn, Ti, V : Each <5
  Ca, K, P: <50
  Al, Pb: 17
  Fe:11
  Pd: 8
  Zn: 5
  Na: 1.03 % (w/w)
- Methylimidazoliumpropyl p-phenylenediamine , Cl, 2HCl (A166) - Batch 000 L 006 (µg/g):
  As, Cd, Hg, Pb, Pd, Sb : Each < 1
  Al, Ba, Co, Cr, Cu, Fe, Mn, Mo, Ni, Se, Sn, Ti, V, Zn : Each <5
  Ca, K, P: <50
  Na : 501

Elemental analysis
Determination of carbon, hydrogen and nitrogen contents were carried out by measure of thermic conductibility (method MO 240 LA 2008).
Determination of oxygen content was carried out by infrared (method MO 238 LA 2008).
Determination of Chloride content was carried out by potentiometry (method MO108 LA 2005)

For Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) batches 008 L 002 and 000 L 006, the experimental elemental analysis values were in accordance with theoretical values.
For batches 002 L 001 and 009 D 003, the experimental elemental analysis values were in accordance with theoretical values, after recalculation.

Ref: Analytical file – Imexine OBL – June 2017

SCCS comments
Percentage content of all of the impurities should be calculated at λmax = 246 nm of the test substance. Quantitative determination of R0011102A (p-phenylenediamine) content by HPLC was carried out at 240 nm.

To prove that the filtration does not interfere with the analysis of the test substance and impurities, a chromatogram obtained from the analysis of the sample before the filtration should be compared ("% area and retention times" of A166 and impurities, number of impurities) to a chromatogram obtained from the analysis of the same sample after filtration and under the same chromatographic conditions. During commenting period, in

Appendix 1, the Applicant has two assays conducted before and after the filtration of the sample, under the same chromatographic conditions showing that the % area of the main product and impurities are comparable in these two assays. SCCS underlines that Applicant should have clearly indicated which of the two assays have been conducted before or after filtration.

Considering MoS calculation based on added information provided by Applicant during the public consultation period (i.e. NOAEL value of 200 mg/kg/day (NOAEL obtained in the 28-day oral toxicity study in rats – OECD 407 (1999)), SCCS agrees on the fact that the presence of the impurity 3-aminopropyl imidazole at a maximum level of 0.1% in Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) presents no or a negligible risk to human health.

Considering complementary information provided by Applicant during the public consultation period, the exposure to the impurity chloroethane to be considered is equal to 100 microgram per day which is below the NSRL of 150 microgram/day. In addition, such an exposure of 100 microgram / day is far below the virtual safe dose (996 microgram/day) calculated based on the LTD10 with a MoE of 10000. SCCS agrees that the presence of the impurity chloroethane at a maximum level of 0.05% in Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) presents a negligible risk to human health.

One of the impurities, 4-aminophenylamine dihydrochloride (synonym p-phenylenediamine dihydrochloride), has already been the subject of an SCCS Opinion (SCCS/1443/11 Revision of 18 September 2012), which raised concerns over its strong skin sensitising potency.

3.1.6 Solubility

Following request from SCCS, data on solubility of Methylimidazoliumpropyl-p-Phenylenediamine, Cl, 2HCl (A166) in water media and in the solvent systems used in various studies have been provided.

According to the Applicant, water was the solvent used in all safety studies performed on Methylimidazoliumpropyl-p-Phenylenediamine, Cl, 2HCl (A166). Accordingly, the solubility was measured in water only.

This study was conducted in compliance with:
- OECD guideline for the testing of chemicals: N° 105 - Water solubility adopted July 27, 1995.
- OECD principles of Good Laboratory Practice.

Test material
Batch R0027494A 008 L 002 (99.9% pure) was used in this study.
A preliminary test for the water solubility was carried out. The water solubility of the test item was estimated to be greater than 1 kg/L in pure water at room temperature (20°C ± 5°C). At this high level of solubility, no pH-dependency could be observed. Based on the guideline the shake flask method was used for the test performance for substances with solubility > 10 mg/L.

Test Principle
Shake flask method
The closed vessels containing (5 g test item, 1 ml solvent) were incubated in a shaking water bath at 30 °C for 24 h, 48 h and 72 h, respectively. Soon after the start of the pre-incubation time, the samples appeared as deep dark brown solution with colourless crystals of 2-5 mm size inside.

At the end of each incubation period the vessels were removed from the water bath and equilibrated for another 24 hours at 20 °C under shaking. The remaining test item was separated by filtration through a 0.45 µm syringe tip filter and analysed by HPLC / UV.

Results
The results showed no tendency to change over time and met all validity criteria, therefore the study is considered to be valid. Based on the analyses, the solubility of the test item in water was determined to be 931 g/L at 20 °C.
The determination of the concentration in the saturated solutions was done under usage of a quadratic regression. The arrangements made to prevent a possible oxidation of the test item (overlay with argon, addition of ascorbic acid in the solvent) seem to be sufficient.

Ref : Wöhr T. (2019)

3.1.7 Partition coefficient (Log P_{ow})

n-Octanol/Water partition coefficient: experimental Log P_{ow} is not determined.
LogP_{ow} *(calculated value)*: -3.3 (ClogP_{ow}, v.5.2).

3.1.8 Additional physical and chemical specifications

- organoleptic properties (colour, odour, taste if relevant): Very light beige to beige powder
- melting point:/
- boiling point:/
- flash point:/
- vapour pressure:/
- density:/
- viscosity:/
- pKa: 2.09 and 5.9 (25°C, ionic strength 0.15 M) for basic equilibria. (Potentiometry, GLpKa Sirius)
- pH: The pH of A166 (Methylimidazoliumpropyl-p-Phenylenediamine, Cl, 2HCl (A166)) diluted at 2% and 10% in water is 1.5 and 1.27/1.24, respectively, as reported in Cannamela (2011) and Cannamela (2015).
- refractive index:/

The following analyses have been performed: UV/visible light absorption, Infra-red spectrometry, 1H NMR and 13C NMR spectrometry, Mass spectrometry. Their results are compatible with the proposed structure of Methylimidazoliumpropyl-p-Phenylenediamine , Cl, 2HCl (A166).

Ref: Cannamela N (2015)

3.1.9 Homogeneity and Stability

The compound is considered to be stable when stored at 4 ± 4 °C in the dark under argon shielded from air and humidity.

Ref: Certificates of Analysis (version 2), Analytical file – Imexine OBL – June 2017; Wöhr T. 2019

Following the request of SCCS, the Applicant has provided information on the stability of Methylimidazoliumpropyl-p-Phenylenediamine, Cl, 2HCl (A166) for dosing formulations used in systemic toxicity studies at 10, 30 and 100 mg/ml in water.

Stability of Methylimidazoliumpropyl-p-Phenylenediamine, Cl, 2HCl (A166) in dose formulation was determined by measuring Methylimidazoliumpropyl-p-Phenylenediamine, Cl, 2HCl (A166) contents using HPLC at different time intervals up to 4 hThe hair dye ingredient Methylimidazoliumpropyl-p-Phenylenediamine, Cl, 2HCl (A166) was found stable in dose formulation for 4h (Raithatha, 2013).

In addition, in the skin penetration study on Methylimidazoliumpropyl-p-Phenylenediamine, Cl, 2HCl (A166) contained in the Applicant's initial safety dossier and performed under typical consumer use conditions (Toner, 2014), an HPLC analysis of the oxidative hair coloring formulation containing Methylimidazoliumpropyl-p-Phenylenediamine, Cl, 2HCl (A166) at 2% (final, on-head concentration) was performed immediately after the application period and 24 hours afterwards. The radiochemical purity and test item concentration was determined by HPLC in the Hair Dye Formulation 24 h post dose to determine the stability of the test item over the live phase of the study.

These analyses yielded Methylimidazoliumpropyl-p-Phenylenediamine, Cl, 2HCl (A166) concentrations of 103.73% and 100.96% of the nominal concentration (2%), respectively, which confirms that Methylimidazoliumpropyl-p-Phenylenediamine, Cl, 2HCl (A166) is stable over a 24-hour period under typical consumer use conditions.

Ref: Raithatha A. (2013), Toner F. (2014)

SCCS comment

The information provided on stability of A166 is not convincing. The use of ascorbic acid and argon for the shaking flask method, and the storage conditions (4 ± 4 °C, in the dark under argon), indicates that the test substance is easily oxidised. The Applicant has provided further information on the stability studies performed for the dose formulation used for toxicity studies, where the dissolution media is distilled water. However, details on the solvent media and the procedure used for preparation of solutions are not fully described in the report.

It is necessary to provide data on the stability of A166 under conditions of use with a selective analytical method. Liquid scintillation counting is not a selective method for this purpose. The stability study was performed before mixing the test substance with the developer, whereas it should be performed after the mixing with the developer. Any degradation products should also be chemically characterised.

The procedure described under 7.13.4 Formulation Stability is different than the procedure described in Appendix 1 - 13.6 Formulation Stability (Toner, 2014),. Any degradation products should be chemically characterised.

3.2 FUNCTION AND USES

The ingredient Methylimidazoliumpropyl-p-Phenylenediamine Cl, 2HCl (A166) is intended to be used in oxidative hair colouring products at on-head concentration of up to 2%.

3.3 TOXICOLOGICAL EVALUATION

The following table provides an overview of the guidelines under which each study submitted in the present dossier was conducted.

STUDY	TEST GUIDELINE	GLP	REFERENCES
In vitro primary cutaneous tolerance test using human reconstructed epidermis (Episkin^{SM})	OECD 439	Yes	Brémond, 2012
In vitro ocular primary irritation test using Bovine Cornea Opacity and Permeability method(i)	OECD 437 modified	Yes	Cannamela, 2011
In vitro ocular primary irritation test using Bovine Cornea Opacity and Permeability method(i)	OECD 437 modified	Yes	Cannamela, 2015
Local lymph node assay in mice	OECD 429	Yes	Verma, 2012
Bacterial reverse mutation test	OECD 471	Yes	Hobson, 2013
Mammalian cell gene mutation test (*hprt* locus) in mouse lymphoma cells	OECD 476	Yes	Lloyd, 2013
Micronucleus test in cultured human lymphocytes	OECD 487	Yes	Watters, 2012
Acute oral toxicity study in rats	OECD 401	Yes	De Jouffrey, 1997
13-week oral toxicity study in rats	OECD 408	Yes	Gohel, 2014
Prenatal developmental toxicity study by the oral route in rats	OECD 414	Yes	Patel, 2014
In vitro percutaneous absorption study using human dermatomed skin	SCCS 1358/10 OECD 428	Yes	Toner, 2014
Bioavailability across intestinal barrier in cultured human intestinal epithelial cells	-	No	Lee, 2015
14-day toxicity study by the oral route in rats	-	Yes	Ujawane, 2013

(i) The *in vitro* ocular primary irritation study using Bovine Cornea Opacity and Permeability method (Cannamela, 2011; Cannamela, 2015) followed a protocol developed internally in order to refine the evaluation of the irritation potential. An additional 30-minute contact period is applied in this study protocol. This method allows a classification of the products into four refined categories not predicted in the OECD 437 guideline. Based on this developed method, IMEXINE® OBL was tested at lower concentration. The concentration tested being the solely deviation from the guideline (10% or 2% instead of 20% as recommended by the guideline), the test was considered to be adequate for safety assessment and regulatory submission.

3.3.1. Acute Toxicity

3.3.1.1 Acute oral toxicity

Guideline: OECD 401
Species/strain: Sprague-Dawley rats
Group size: One group of 3 males and 3 females
Test substance: C16778
Batch: RF006
Vehicle: Purified water
Dose levels: 500 mg/kg (volume of 10 ml/kg)
Administration: oral (gavage), single
GLP: In compliance
Study period: 2 June 1997 - 4 August 1997

The acute oral toxicity of Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) was evaluated following single administration to fasted rats at 500 mg/kg.

Three Sprague-Dawley (SD) rats per sex were used in this study to assess the acute oral toxicity of Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166).

On a day designated as day 1, 3 overnight fasted SD rats per sex received a single oral (gavage) dose of 500 mg/kg Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) in purified water (10 mL/kg). Animals were observed at least daily for clinical signs and twice daily for mortality. Body weights were recorded on day 1 just before treatment and on days 5 and 8. Animals found dead or killed prematurely and all surviving animals killed at the end of the study were subjected to macroscopic examination. Organs showing gross anomalies were sampled and preserved, but no histopathological examination was performed. Neither mortality nor clinical signs were observed during the study. Macroscopic examination of the main organs of the animals revealed no apparent abnormalities.

Conclusion
Under the conditions of this study, no clinical signs and no deaths were observed after a single oral administration of Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) at the dose level of 500 mg/kg in rats. The oral LD50 was higher than 500 mg/kg.

Ref: De Jouffrey S (1997)

SCCS comments
Animals were observed until day 8 instead of for 14 days, but since no mortality was registered, it can be deduced that the oral LD50 is higher than 500 mg/kg b.w.

3.3.1.2 Acute dermal toxicity

/

3.3.1.3 Acute inhalation toxicity

/

3.3.2 Irritation and corrosivity

3.3.2.1 Skin irritation - *In vitro* Primary Cutaneous Tolerance

Guideline: ECVAM validated protocol (ESAC Statement 2007)
Test system: 96 reconstructed human epidermis Episkin^{SM} samples (0.38 cm²)
Number of replicates: Triplicate tissues for each item
Test substance: R0027494A
Test item: 10% (w/w) R0027494A in water
Batch: R0027494A 000 L 006
Purity: >95%
Dose: 10 µl
Treatment period: 15± 1 minutes
Post-treatment incubation time: 42 h± 1h
Positive control: 5% aqueous solution of SDS
Negative control: PBS
Direct interaction with MTT: Positive
Colouring potential test item: Positive
GLP: in compliance
Study period: November 2011 – May 2012

Test Procedure
The test item diluted at 10% (w/w) in water was evaluated in 3 different batches of a reconstructed human epidermis model. After preliminary tests (dilution and staining tests), the coloured test item was tested according to the specific colouring protocol.
The test item, negative control (PBS+), positive control (5% aqueous solution of Sodium Dodecyl Sulfate), solvent control (water), and a dead epidermis negative control (PBS+) were tested in triplicate. Two additional negative controls (one dead and one living tissue) were used which followed the same treatment as the negative control (PBS+) but underwent an additional MTT incubation period.
Six additional tissues (three living and three dead tissues) were used and followed the same treatment as the other tissues (except for the MTT incubation period).
These tissues as well as the additional negative control (PBS+) were used as specific controls in order to quantify the extent of Non-Specific Colour due to the colouring chemical interactions with the tissue.

10 µl of Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) diluted at 10% and 10 µL of different controls were applied onto the epidermis using a positive displacement pipette. After 15 minutes' treatment period at room temperature, tissues were rinsed with PBS+, and then epidermis were transferred in 2 ml/well of fresh maintenance medium and incubated for 42 hours +/- 1 hour at 37°C.

MTT test:
At the end of the 42 hours +/- 1-hour treatment period, each epidermis unit was transferred to the wells of a 12 well plate containing a dye solution (MTT) except for the negative control and the test item-treated epidermis without MTT which were transferred into a 12 well plate containing fresh medium. Plates were incubated for 3 hours to 3 hours 15 minutes at 37°C. At the end of the incubation period, a biopsy of the entire epidermis was taken. For all tissues with the test item, the superficial epidermis layer (containing most of the remaining color) was removed and discarded. The epidermis was separated from the collagen matrix and both were transferred into a tube containing 500 µL of acidified isopropanol. Formazan crystals were extracted and stirred to homogenize the solution. 2 x 200 µL of each extract were transferred onto a 96 well plate and the optical density (OD) was measured at 570 nm versus acidified isopropanol.

Determination of IL-1α concentrations in the culture medium:
IL-1α released in the culture medium was determined by a classic quantitative sandwich enzyme immunoassay technique. The optical density (OD values related to the IL-1α amount) was measured at 450 nm.

The test item is predicted to be non-irritant when mean viability value is above 50% and final IL-1α release is below 50 pg/mL. The test item is predicted to be irritant when mean viability value is lower than (≤) 50% or final IL-1α release is above (≥) 50 pg/mL.

Results
The mean viability value for the test item diluted at 10% was 76.2±12.8% and the final IL-1α release was 1.6±2.9 pg/mL.

Conclusion
Under the conditions of this study, Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) at the concentration of 10%(w/w) in water is considered to be non-irritant to the skin.

Ref: Brémond C. (2012)

3.3.2.2 Eye irritation

Bovine corneal opacity and permeability method (BCOP)
The acute ocular irritation potential of Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) diluted at 2% and 10% in water was evaluated in an in vitro Bovine Cornea Opacity and Permeability (BCOP) test [Cannamela N. (2015) ; Cannamela N. (2011)].

	Cannamela N. (2011)	Cannamela N. (2015)
Guideline	OECD 437 modified	OECD 437 modified
Test system	Bovine eyes (from cattle aged less than 12 months)	Bovine eyes (from cattle aged less than 12 months)
Group size	3 corneas	3 corneas
Test substance	R0027494A	R0027494A
Test item	10% (w/w) R0027494A in distilled water	2%(w/w) R0027494A in distilled water
Batch	R0027494A 000 L 006	R0027494A 009 D 003
Purity:	>95% (UV detection)	98% pure
Dose applied:	750 ± 8µl	750 ± 8µl
Treatment period:	30 ± 5 minutes, 4 hours ± 10 minutes	30 ± 5 minutes, 4 hours ± 10 minutes
Post-treatment Incubation time	2 hours for 30 minutes incubation time, none for 4 hours	2 hours for 30 minutes incubation time, none for 4 hours
Negative control	Nutritive medium (MEM, sodium bicarbonate, distilled water)	Nutritive medium (MEM, sodium bicarbonate, distilled water)
Positive control	0,5% (w/w) Cetyl Trimethylammonium Bromide (CTAB)	CTAB at 0.5% in water, and 20% imidazole solution w/w in NaCl 0.9% for the 30 minutes and 4 hours contact time, respectively
GLP	In compliance	In compliance
Study period	Experiment from 24 October 2011 to 27 October 2011 Study completion : 15 November 2011	22 January 2015 to 29 January 2015

Test Procedure
Bovine eyes (from cattle less than 12 months old) were collected at slaughterhouses and prepared within 4 hours of collection. Eyes that were too big or were presenting defects were rejected. After the pre-incubation and equilibration period of the corneas at 32 ± 1°C for 1h ± 10 minutes, 750 ± 8 µL of the test item and controls were applied onto the corneas. The treatment period of 30 ± 5 minutes and 4 hours ± 10 minutes was followed by three rinsing steps and visual examination of rinsing efficiency. The corneal opacity and permeability were measured. Corneas were incubated with 0.5% fluorescein solution for 90 ± 5 minutes at 32 ± 1°C; corneal permeability was performed by measuring optical density at 490 nm.
The following validity criteria were applied in the study:
- opacity of the negative control (nutritive medium) corneas: between 0 and 10
- O.D. of the negative control (nutritive medium) corneas: lower than 0.100
- O.D. of the fluorescein solution to 5 µg/mL: between 0.850 and 0.940
- score CTAB to 0.5%:
    - 30 minutes: value between 85.0 and 105.0
    - 10 minutes: value between 25.0 and 40.0.

Results
When tested at the concentration of 10%, the corneal score (a combination of opacification and permeability measurements) obtained after an exposure period of 30 minutes and 4 hours contact was 5.5±0.8 and 96.7±0.7, respectively. Accordingly, Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) at a concentration of 10% was considered to be moderately irritant to eyes.

Furthermore, Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) was tested at the use concentration of 2%. The corneal score obtained after an exposure period of 30 minutes and 4 hours was 0.7±0.8 and 17.5±0.6, respectively. Accordingly, Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) at a concentration of 2% was considered to be non-irritant to slightly irritant to eyes.

Ref: Cannamela N. (2011), Cannamela N (2015)

SCCS comment
The BCOP assay is an *in vitro* method that can be used to identify ocular corrosive and severe irritants, but not moderate irritants. Considering that the test item is brown coloured and that colouration of epithelial tissue by the test item has been reported in the *in vitro* skin irritation test, it is expected that colouration of the cornea might have occurred.

Even though Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) diluted to 2% and 10% w/w in water has a very low pH (reported to be < 1.5) which may lead to corrosive effects, the BCOP assay did not indicate severe eye irritation. Under the conditions of this study, a mild to moderate eye irritation potential of the test item at 2% and 10% concentrations cannot be excluded.

3.3.3. Skin sensitisation

Local Lymph Node Assay (LLNA)

Guideline:	OECD 429, EC B.42
Species/strain:	Female CBA/J mice
Group size:	4 mice per group (main study), 2 mice per group (preliminary assay)
Test substance:	Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166)

Batch:	R0027494A 002 L 001
Purity:	>95%
Vehicle:	Pluronic® L92 1% in water
Concentration:	1, 5, 25 and 50% (w/v)
Positive control:	alpha-hexylcinnamaldehyde (HCA) 25%(v/v) in water with 1% Pluronic® L92
Vehicle control:	Pluronic® L92 1% in water
GLP:	In compliance
Study period:	Febr – Sept 2012

The skin sensitising potential of Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) was evaluated in a Local Lymph Node Assay (LLNA) in mice.
Animals (28 mice) were separated in 7 groups (4 mice/group) consisting of:

- 5 treated groups receiving Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) at 1, 5, 10, 25 or 50% (w/v) in 1% Pluronic L-92 (vehicle). Due to unsatisfactory solubility tests with several recommended vehicles, Pluronic L-92 was selected on the basis of a solubility study revealing 50% (w/v) Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) as the maximal technically feasible concentration.
- A negative control group receiving the vehicle (1% Pluronic L-92) alone
- A positive control group receiving alpha-hexylcinnamaldehyde (HCA) at 25% (v/v) in 1% Pluronic L-92

The test substance Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) and 1% Pluronic L-92 or HCA were applied on the ears (25 µL per ear) of mice for three consecutive days designated as days 0, 1 and 2. After 2 days of resting (day 5), mice received a single intravenous injection of tritiated methyl thymidine (^{3}H-TdR). Lymph nodes draining the application sites (auricular nodes) were sampled, pooled per group, and the proliferation of lymphocytes was evaluated by measuring the incorporation of ^{3}H-TdR. The values obtained were used to calculate stimulation indices (SI). The irritant potential of the test item was assessed by measuring ear thickness on days 0, 2 and 5.

Results
All validity criteria were fulfilled and the study was therefore considered to be valid. In particular, a SI value of 8.04 was obtained with the positive control HCA.
The threshold value of 3 for positive results was exceeded at 5% with a dose-response effect (SI values of 2.69, 7.99, 10.77, 12 and 15.91 at 1%, 5%, 10%, 25% and 50% respectively) and the EC3 value was calculated to be 1.23%.

Conclusion
Under the conditions of this study, Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) induced delayed contact hypersensitivity. According to the EC3 value calculated (1.23%), Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) was considered to have a moderate sensitising potential.

Ref: Verma 2012

SCCS comment
SCCS considers Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) as a strong skin sensitiser, based on the EC3 value of 1.23% observed in the LLNA (see SCCP, 2005)

3.3.4 Toxicokinetics

3.3.4.1 Dermal / percutaneous absorption

***In Vitro* Percutaneous Absorption of Radiolabelled R0027494A Through Human Skin.**

Guideline:	OECD 428 (2004); OECD 28 (2004), SCCS 1358/10 (2010), COLIPA Cosmetic Ingredients: Guidelines for Percutaneous Absorption/Penetration (1997)
Test system:	Split-thickness human skin 350-400 µm, from 5 donors aged 40 – 56 years (1 breast, 4 abdomen)
Membrane integrity:	Checked by a tritiated water method
Replicates:	12 skin samples from 4 different donors
Test substance:	R0027494A (non-labelled); [^{14}C]-R0027494A (labelled)
Batch:	R0027494A 009 D 003 (non-labelled); CFQ41883 (labelled)
Purity:	98% (non-labelled material); 96.8% (radiochemical purity by HPLC)
Test item:	Hair dye formulation containing 4% (w/w) [^{14}C]-R0027494A
Dose applied:	20 mg/cm^2 of the test formulation
Exposure area:	0.64 cm^2
Exposure period:	30 minutes
Sampling period:	24 hours (every hour between 0-6h, then every 2 hour)
Receptor fluid:	Phosphate buffered saline (PBS)
Solubility in receptor fluid:	100g/l in water
Mass balance analysis:	Provided
Tape stripping:	Yes (20)
Method of Analysis:	Liquid Scintillation Counting (LSC)
GLP:	In compliance
Study period:	March– April 2014

The percutaneous absorption of 2% Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) was investigated *in vitro* in a oxidative hair dye formulation, in the presence of hydrogen peroxide by using human skin preparations. A total of 12 samples of human skin obtained from 4 different donors were used for each test group. Mean values were calculated from all valid skin samples (n=12, four donors). The integrity of the skin was demonstrated prior to application by a tritiated water method. Only skin samples within the acceptable range of <0.6% were used.

Split-thickness human skin membranes were mounted into flow-through diffusion cells, receptor fluid was pumped underneath the skin at a flow rate of 1.5 mL/h ± 0.15 mL/h. The skin surface temperature was maintained at 32°C ± 1°C throughout the experiment. Test item at 2% (w/w) was applied at an application rate of ca 20 mg/cm^2 for 30 minutes.

Absorption of Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) was evaluated by collecting receptor fluid in 60 min fractions from 0 to 6 h post dose, then in 2-hourly fractions from 6 to 24h post dose. The stratum corneum was removed by tape stripping and the remaining skin was divided into exposed and unexposed skin. The exposed epidermis was then heat separated from the dermis. The radioactivity of radiolabelled Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) was quantified by liquid scintillation counting.

Results
The majority of the test substance was removed in the skin wash after the 30-minute exposure (97.52%). A further 0.07% of the applied dose was removed at 24 h (donor wash). The total dislodgeable dose was 97.59% of the applied dose. The mean total

unabsorbed dose was 99.35% of the applied dose. This consisted of the dislodgeable dose, unexposed skin (0.01%) and the radioactivity associated with the stratum corneum (1.74%). The first five tape strips contained 1.12% of the applied dose. There was a steady decrease in the recovery of radioactivity associated with the stratum corneum. Tapes 6-10, 11-15 and 16-20 contained 0.40%, 0.16% and 0.07%, respectively. Those are not considered to be dermally absorbed and thus do not contribute to the systemic dose. Where any epidermis was removed during tape stripping this tape strip value was added to the epidermis and not to the stratum corneum value.
The absorbed dose (0.06%) was the sum of the receptor fluid (0.05%), the receptor rinse (<0.01%) and the receptor wash (0.01%). Dermal delivery: 0.29% (1.15 ± 0.77 µg equiv./cm^2) was the sum of the absorbed dose and the epidermis (0.19%) and dermis (0.04%). The mean mass balance was 99.63% of the applied dose after 24 h.

The table below shows a summary of the mean test results:

Test Preparation	Test Preparation
Target [^{14}C]-R0027494A Concentration in Formulation (%, w/w)	4
Actual [^{14}C]- R0027494A Concentration in Formulation (%, w/w)	3.98
Target [^{14}C]- R0027494A Concentration in Test Preparation (%, w/w)	2
Actual [^{14}C]- R0027494A Concentration in Test Preparation (%, w/w)	1.95
Target Application Rate of Test Preparation (mg/cm^2)	20
Actual Application Rate of Test Preparation (mg/cm^2)	20.4
Total Number of Donors	4
Total Number of Replicates Dosed	3
Total Number of Replicates Contributing to Mean ± SD Data	12
(% Applied Dose)	(Mean ± SD)
Dislodgeable Dose	97.59 ± 1.47
Unabsorbed Dose *	99.35 ± 1.33
Absorbed Dose **	0.06 ± 0.02
Dermal Delivery ***	0.29 ± 0.19
Mass Balance	99.63 ± 1.17
(µg equiv./cm^2)	(Mean ± SD)
Dislodgeable Dose	388.63 ± 5.79
Unabsorbed Dose *	395.60 ± 4.87
Absorbed Dose **	0.23 ± 0.07
Dermal Delivery ***	1.15 ± 0.77
Mass Balance	396.75 ± 4.35

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

Conclusion
Under the experimental conditions of the study, the mean dose of Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) considered to be penetrated following application of a typical oxidative hair colouring formulation containing this ingredient at 2% was estimated to be 1.15 ± 0.77 µg equiv./cm^2 (0.29 ± 0.19% of the applied dose).

Ref: Toner F. (2014)

SCCS comment
Correct calculation of the standard deviation is 0.74 µg/cm^2.

In accordance with the SCCS Notes of Guidance, the mean + 1 SD of dermal absorption value, i.e. 1.89 µg/cm^2 (0.48%), will be used for MoS calculation.

3.3.4.2 Other studies on toxicokinetics

Study to Investigate the Permeability of the Test Compound, R0027494A, in Caco-2 Cells

Guideline:	Study performed according to the recommendations by ECVAM and is generally recognized as scientifically relevant (ECVAM, DB-ALM Protocol n° 142)
Species/strain:	Human intestinal epithelial cells line Caco-2
Test substance:	Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166)
Batch:	R0027494A 009 D 003
Purity:	98% pure
Concentration:	10 µM in HBSS (pH 7.4) with a final DMSO concentration of 1%
GLP:	non-GLP conditions

Test Procedure
The test item was prepared at 10 µM in HBSS (pH 7.4) with a final DMSO concentration of 1%. The permeability from the apical (A, pH 7.4) to the basolateral (B, pH 7.4) side was investigated at 37°C in 96-well Multiscreen plates with shaking for a 60 min contact period. Analysis of the donor (apical) and receiver (basolateral) samples was done by means of the LC-MS/MS and the apparent permeability coefficient (P_{app}) of the test item and its recovery were calculated for two independent experiments. Lucifer yellow was used to demonstrate the integrity of the cell monolayer. Only monolayers revealing a permeability of less than 1.5 x 10^{-6} cm/sec were used. Propranolol (highly permeable~90%), atenolol (moderately permeable~50%), and talinolol (P-glycoprotein substrate) were analyzed concurrently to demonstrate the validity of the assay.
According to the laboratory's classification system, a low permeability is considered for test items revealing a P_{app} = 0.2 - 2 x 10^{-6} cm/sec. A P_{app} of 2 - 20 x 10^{-6} cm/sec and a P_{app} = 20 – 45 x 10^{-6} cm/sec classify a substance to have a moderate and a high permeability, respectively. As recommended by FDA, atenolol (50% absorption in humans) was used as the low permeability reference compound and propranolol (90% absorption in humans) was used as the high permeability reference compound.

Results
The total recovery for the reference substances ranged from 76.5 to 94.4% and was 93.4% for Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166).
The figures for the reference substances propranolol (P_{app} = 34.0 x 10^{-6} cm/sec) and atenolol (P_{app} = 0.39 x 10^{-6} cm/sec) were well within the acceptance range for these compounds of 20 - 45 x 10^{-6} cm/sec and P_{app} 0.2 - 2 x 10^{-6} cm/sec, respectively, and demonstrated the validity of the assay.
Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) revealed a P_{app} of 5.73 x 10^{-6} cm/sec and thus, was classified to be of moderate permeability, indicating a moderate absorption from the gastro-intestinal tract.

Conclusion
A mean permeability in human intestinal epithelial cells (Caco-2) of 5.73 x 10^{-6} cm/sec was obtained with Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) which indicates a moderate permeability. As the absorption from the gastro-intestinal epithelium is considered to be the limiting factor of the uptake through the gastro-intestinal tract, the moderate permeability observed in this assay points to a moderate absorption of Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) after oral administration.

Ref: Lee S. (2015)

SCCS comment
A moderate permeability observed in this study supports the default value of 50% for oral bioavailability, which will be used for the calculation of the MoS.

3.3.5 Repeated dose toxicity

3.3.5.1 Repeated dose 14 Days oral toxicity

Species/strain:	Wistar rats
Group size:	5 per dose per sex
Test substance:	R0027494A, Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166)
Batch:	008 L 002
Purity:	99.9%
Vehicle:	distilled water
Dose levels:	0 (G1), 30 (G2), 100 (G3), 300 (G4) and 1000 (G5) mg/kg b.w.
Dose volume:	10 mL/kg
Route:	oral
Administration:	oral gavage of 14 consecutive days
GLP:	yes
Study period:	May 11, 2012 to October 10, 2103

Method
Total 25 male and 25 female Wistar were randomly divided into five groups. Four dose levels of R0027494A were tested: 30 (G2-Low dose), 100 (G3-Mid dose-I), 300 (G4-Mid dose-II) and 1000 (G5 High dose) mg/kg b. wt./day. A concurrent control group of rats was given distilled water only. The fix dose volume of 10 mL/kg body weight was used. The dose formulations were prepared by dissolving the test item in distilled water.

All rats were observed once a day for clinical signs and twice a day for mortality and morbidity throughout the study period. Body weight and food consumption of individual animals were determined twice weekly throughout the study. Haematological and biochemical analyses were performed on blood samples of all rats at the end of the treatment period.

All the surviving rats were sacrificed by carbon dioxide asphyxiation and subjected to gross pathological examination at the end of the treatment period. Absolute organ weights were recorded and relative organ weights were calculated for the organs defined in study plan.

Results
No mortality was observed from any of the groups (G1 to G5) during the study period.
All animals belonging to groups G1 and G5 were found normal throughout the study period
Some changes were observed in mean body weight gain of rats belonging to group G5 and in food consumption of female rats from groups G2, G3, G4 and G5 when compared to the G1 rats. These changes were limited, not dose related in female and therefore of uncertain biological significance.
No treatment-related alterations were observed in haematology and clinical chemistry parameters of R0027494A treated groups when compared with the control group.
No treatment related change in polychromatic to total erythrocytes ratio (P/E) was observed in all the R0027494A treated groups as compared to control group.
No external and internal gross lesions were observed across all the groups.
No treatment related microscopic changes were observed in rats treated with R0027494A at 1000 mg/kg b.wt./day.

Conclusion
Based on the results of the study, it is concluded that, with the exception of a slight decrease in body weight gain at the highest dose in males and a non dose-related decrease in body weight gain at all doses in females with a decreased food consumption in the two highest dosed groups, R0027494A did not produce any adverse effect up to the dose level of 1000 mg/kg b.wt./day when administrated through oral gavage for 14 consecutive days in Wistar rats under the conditions and the procedures followed in the present study.

Consequently, the dose level of 1000 mg/kg b.wt./day can be selected as the highest dose to be tested for further repeated toxicity study.

Ref: Jawane D (2013)

SCCS comment
The study was performed as a range-finder for an oral 90 d repeat-dose toxicity study. The results of this study support a low acute toxicity of Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166).

3.3.5.2 Sub-chronic (90 days) oral toxicity

Guideline:	OECD 408 (1998)
Species/strain:	Wistar rats
Group size:	10 males and 10 females
Test substance:	R0027494A, Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166)
Batch:	009 D 003
Purity:	99.9 %
Vehicle:	distilled water
Dose levels:	0 (G1), 100 (G2), 300(G3), 1000 (G4) mg/kg b.w.
Dose volume:	10 mL/kg b.w.
Route:	oral
Administration:	gavage – 13 weeks
GLP:	yes
Study period:	December 11, 2012 to November 14, 2014

The experimental phase of the 13-week oral toxicity in rats was conducted from December 2012 to March 2013, and the corresponding study report was completed in 2014

Test Procedure
The subchronic toxicity of Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) was investigated in Wistar rats (10/sex/group) after daily oral gavage at 0, 100, 300 or 1000 mg/kg/day in distilled water (10 mL/kg) for 13 weeks. These dose levels were selected on the basis of the results of a preliminary 14-day study (see section 3.3.5.1). Evaluations and measurements included twice daily mortality checks and clinical observations, weekly body weight, food intake and neurobehavioral observation, ophthalmoscopy prior to dosing and at the end of the treatment period, neurotoxicological evaluation during week 12, haematology, blood clinical chemistry and urinalysis (week 13). Neurobehavioral observations were conducted on each rat once prior to treatment initiation and at weekly intervals thereafter. At the end of the treatment period, surviving animals were sacrificed and subjected to macroscopic examination; selected organs were weighed, and a wide range of organs/tissues were preserved. Microscopic examination was performed for specified tissues/organs from control and high dose rats killed at the end of the dosing period, as well as for any gross anomaly.

Results
The analysis of the dose formulations administered during the study showed that the concentrations given were within the appropriate range.

At 100 and 300 mg/kg bw, the test item Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) did not induce any relevant treatment-related changes.

At 1000 mg/kg/day in males, statistically significant decreases in mean body weights were observed during week 9, 10, 11 and 13 and in mean body weight changes (throughout treatment period). There were no apparent abnormalities in ophthalmology, neurobehaviour and functional observational battery.
In males of the highest dose, relative weights of liver, spleen, brain, kidneys and adrenals were statistically significantly higher when compared to controls and absolute weights of thymus and epididymides were statistically significantly lower when compared to controls. In females at the highest dose a significant increase was observed in absolute and relative weights of livers and ovaries and in relative weights of adrenals.
The weight changes were not accompanied by histopathological findings but considered test-item related by the study authors. Treatment-related changes were observed in clinical chemistry in males and females at the highest dose (significantly lower blood glucose, alkaline phosphatase and albumin concentrations levels; significantly higher blood phosphorus concentrations). Females at the highest dose also had significantly higher blood AST concentrations and significantly lower blood sodium and chloride concentrations. Furthermore, haematology revealed significantly higher Hb, MCH and MCHA values and significantly lower APTT (Activated Partial Thromboplasin Time) and PT (prothrombin time) values in males at the highest dose and significantly lower PT values in females at 300 and 1000 mg/kg bw/d. These effects were not considered treatment-related by the study authors.

Conclusion
Thus, under the conditions of the study, the NOEL (No Observed Effect Level) of this 90-day repeated oral toxicity study with Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) in Wistar rats was defined at 300 mg/kg /day.

Ref: Gohel D (2014), Ujawane D (2013)

SCCS comment
Based on limited adverse effects (changes in clinical chemistry and haematology at the highest dose), the SCCS derives a NOAEL of 300 mg/kg bw/d from this study.

3.3.5.3 Chronic (> 12 months) toxicity

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3.3.6. Reproductive toxicity

3.3.6.1 Fertility and reproduction toxicity

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3.3.6.2 Developmental Toxicity

Guideline:	OECD 414 (2001)
Species/strain:	Wistar rats
Group size:	25 mated females per group
Test substance:	R0027494A, Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166)
Batch:	009 D 003

Purity:	99.9%
Vehicle:	distilled water
Dose levels:	0 (G1), 100 (G2), 300 (G3), 1000 (G4) mg/kg b.w. from gestional days (GD) 6 to 19
Dose volume:	10 mL/kg b.w.
Route:	oral
Administration:	gavage
GLP:	Yes
Study period:	December 11, 2012 to October 30, 2014 The experimental phase of the Prenatal development toxicity by the oral route in rats was conducted from December 2012 to January 2013, and the corresponding study report was completed in 2014

Test Procedure
The potential effects of Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) on pregnant rats and embryo-foetal development were evaluated through daily oral gavage in which mated Wistar female rats (25/group) were dosed at 0, 100, 300 or 1000 mg/kg/day during the sensitive period of organogenesis from gestation day 6 to day 19 [the day of mating was designated as Gestation Day 0 (GD 0)]. The test item was dissolved in water and given at 10 mL/kg. These dose levels were selected on the basis of the results of a preliminary 14-day repeated toxicity study performed at 100, 300 and 1000 mg/kg/day where no adverse effects were observed [Ujawane 2013]. Maternal evaluations and measurements included daily observation of clinical signs and body weight/food intake at designated intervals. The dams were killed on GD 20 and subjected to macroscopic examination. Usual litter parameters were recorded and foetuses were sexed, weighed and submitted to external examination. About one half of the foetuses were also examined for soft tissue anomalies, and remaining foetuses were examined for skeletal anomalies.

Results
The analysis of the dose formulations administered during the study showed that the concentrations given were within the appropriate range.
No mortality was observed in dams up to the dose level of 1000 mg/kg/day. Lethargy was observed in animals given 300 mg/kg bw/d and above, associated with weakness, hypoesthesia and somnolence at 1000 mg/kg bw/d. Decreases in mean body weight, mean body weight changes, corrected body weight gain and food consumption were observed at the same dose level when compared to the control group. At 100 and 300 mg/kg/day, no clinical signs of toxicological relevance were observed in dams. External und visceral examination of dams did not reveal any lesion of pathological significance. Mean numbers of corpora lutea, implantations, live foetuses, dead foetuses and mean rates of pre-implantation losses, post-implantation losses, live foetuses and dead foetuses in treated animals were not different from control. Slight increase in resorptions was also observed in treated dams at 300 and 1000 mg/kg/day but not statistically significant.
Mean number of litters and fetal body weights in treated animals were not different from controls. There were no gross findings, no external malformations and no skeletal findings in foetuses of treated animals. Visceral examination indicated isolated gross lesions and were considered incidental by the study authors. Thus, there were no indications for a teratogenic potential.

No significant changes in foetal parameters were observed at any dose level of Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) when compared to the control group.

Conclusion
On the basis of the results obtained in the present study, the No Observed Adverse Effect Levels (NOAEL) for maternal and developmental toxicity of Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) were set at 100 mg/kg/day and 1000 mg/kg/day,

respectively. Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) was considered to have no teratogenic potential.

Ref: Patel MV (2014)

SCCS comment
Methylimidazoliumpropyl phenylenediamine, Cl, 2HCl (A166) is not teratogenic in rats.

A NOAEL of 1000 mg/kg bw/d is derived for developmental toxicity.

A NOAEL of 100 mg/kg bw/d is derived for maternal toxicity due to changes in lethargy at 300 and 1000 mg/kg bw/d and weakness, hypoaesthesia and somnolence and decreased mean body weight gain and mean percent body weight and of absolute uterine weight and uterine weight corrected to body weight at 1000 mg/kg bw/d.

3.3.7 Mutagenicity / genotoxicity

3.3.7.1 Mutagenicity / genotoxicity *in vitro*

Bacterial Reverse Mutation Assay

Guideline:	OECD 471 (1997)
Species/Strain:	*Salmonella typhimurium* TA1535, TA1537, TA98, TA100 and TA102
Replicates:	two independent experiments in the absence and presence of metabolic activation (S9 mix prepared from the livers of rats given Aroclor 1254)
Test substance:	Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166)
Batch:	R0027494A 008 L 002
Purity:	99.9%
Solvent:	deionised water
Concentrations:	5, 15.81, 50, 158.1, 500, 1581 and 5000 µg/plate in experiment 1, both in the absence and presence of S9 mix 20.48, 51.2, 128, 320, 800, 2000 and 5000 µg/plate in experiment 2, both in the absence and presence of S9 mix
Treatment:	Experiment I direct plating incorporation method, Experiment II without S9 MIX direct incorporation method with S9 mix the pre-incubation method (20 min).
GLP:	in compliance
Study period:	June 2012 – January 2013

Test Procedure
The test item Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) was evaluated in two independent experiments in the absence and presence of metabolic activation (S9 mix prepared from the livers of rats given Aroclor 1254). The experiments were conducted according to the direct plating incorporation method, apart from the second test with S9 mix which was performed according to the pre-incubation method. Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) was freely soluble at 50 mg/mL in water.

Toxicity was evaluated for range of concentrations up to the prescribed maximum concentration of 5000 µg/plate on the basis of a reduction in the number of revertant colonies and/or clearing of the bacterial background lawn. Negative and positive controls were in accordance with the OECD guideline. Known mutagens were used as positive controls, and cultures treated with purified water (solvent) were used as negative controls. Three plates per treatment condition were used except for negative control (five plates).

Results
All solvent and positive controls gave counts of revertants within expected ranges, and the experiments were therefore considered to be valid.
The test article was completely soluble in the aqueous assay system at all concentrations treated, in both experiments.

Experiment 1 : Evidence of toxicity in the form of a slight thinning of the background bacterial lawn and/or a marked reduction in revertant numbers was observed following treatments at 1581 and/or 5000 µg/plate in strains TA98, TA1535, TA1537 and TA102 in the absence and presence of S-9 and strain TA100 in the absence of S-9 only.

Experiment 2 : Evidence of toxicity in the form of a slight thinning of the background bacterial lawn and/or a reduction in revertant numbers was observed following treatments at 2000 and/or 5000 µg/plate in strains TA98, TA1537 and TA102 in the absence and presence of S-9 and strain TA1535 in the absence of S-9 only.

When compared to controls, no increases in the number of revertants were observed after treatment with Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166), either in the absence or presence of S9 mix.

Conclusion
Under the conditions of this study, Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) was not mutagenic in *Salmonella typhimurium* strains TA1535, TA1537, TA98, TA100 and TA102 neither in the presence nor absence of metabolic activation.

Ref: Hobson S (2013)

SCCS comment
In experiment 1, R0027494A was clearly bacteriotoxic in low concentrations in the strain TA1537 and in the absence of S9-mix.

Bacterial Reverse Mutation assay on strain TA1537 in the absence of metabolic activation

Guideline:	OECD 471 (1997)
Species/Strain:	*Salmonella typhimurium* TA1537
Replicates:	two independent experiments in the absence of metabolic activation (S9 mix prepared from the livers of rats given Aroclor 1254)
Test substance:	Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166)
Batch:	R0027494A 008 L 002
Purity:	99.9%
Solvent:	water for irrigation (purified water),
Concentrations:	stock concentration at 50 mg/mL Experiment 1: 5, 16, 50, 160, 500, 1600 and 5000 µg/plate in the absence of S9 mix Experiment 2: 160, 300, 625, 1250, 2500, and 5000 µg/plate in the absence of S9 mix
Treatment:	Experiment I direct plating incorporation method, Experiment II with the pre-incubation method (20 min).
Positive control:	9-aminoacridine (AAC) at 50 µg/plate
GLP:	in compliance
Study period:	December 4, 2018 – February 22, 2019

Material and methods
R0027494A was tested for mutation (and toxicity) in one strain of Salmonella typhimurium (TA1537), in two separate experiments, at the concentrations detailed above, using triplicate plates without S-9 for test article, vehicle and positive controls.
As the results of Experiment 1 were negative, treatments in Experiment 2 included a pre-incubation step. Quantities of test article, vehicle or positive control, bacteria and buffer solution were mixed together and incubated for 20 minutes at 37±1°C, with shaking, before the addition of 2 mL molten agar at 45±1°C. Plating of these treatments then proceeded as for the normal plate-incorporation procedure.

Results
Experiment 1 treatments of the tester strain were performed in the absence of S-9, using final concentrations of R0027494A at 5, 16, 50, 160, 500, 1600 and 5000 µg/plate, plus vehicle and positive controls. Following these treatments, evidence of toxicity in the form of a slight thinning of the background bacterial lawn was observed at 5000 µg/plate.
Experiment 2 treatments of the tester strain were performed in the absence of S-9. The maximum test concentration of 5000 µg/plate was retained. Narrowed concentration intervals were employed covering the range 160 to 5000 µg/plate, in order to examine more closely those concentrations of R0027494A approaching the maximum test concentration and considered therefore most likely to provide evidence of any mutagenic activity. In addition, all treatments were further modified by the inclusion of a pre-incubation step. In this way, it was hoped to increase the range of mutagenic chemicals that could be detected using this assay system. Following these treatments, evidence of toxicity in the form of a slight thinning of the background bacterial lawn was observed at 5000 µg/plate.
The test article was completely soluble in the aqueous assay system at all concentrations treated, in each of the experiments performed. Intense colouration caused by the test article was observed on the test plates at 5000 µg/plate in Experiments 1 and 2.
Following R0027494A treatments of the test strain in the absence of S-9, no increases in revertant numbers were observed that were ≥3-fold the concurrent vehicle control in strain TA1537. This study was considered therefore to have provided no evidence of any R0027494A mutagenic activity in this assay system.

Conclusion
It was concluded that R0027494A did not induce mutation in a histidine-requiring strain (TA1537) of Salmonella typhimurium when tested under the conditions of this study. These conditions included treatments at concentrations up to 5000 µg/plate (the maximum recommended concentration according to current regulatory test guidelines, which was a toxic concentration) in the absence of an endogenous metabolic activation system (S-9).

Ref: Lloyd M. (2019)

SCCS comment
The bacteriotoxicity of Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166), batch R0027494A occurred at the highest tested concentration of 5000 µg/plate in the S. typhimurium TA1537 strain.

In vitro **Mammalian Cell Gene Mutation Test in Mouse Lymphoma cells (*Hprt*-locus)**

Guideline:	OECD 476 (1997)
Cells:	L5178Y cells (MLA)
Replicates:	duplicate cultures in three independent experiments
Test substance:	Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166)
Batch:	R0027494A 008 L 002
Purity:	~99.9% pure

Solvent:	deionised water
Concentrations:	Experiment 1 In the absence of S9 mix: 2.5, 5, 10, 20, 40, 60, 80, 100, 150 and 200 µg/mL (cytotoxicity: 18% relative survival at 200 µg/mL) In the presence of S9 mix: 100, 200, 400, 800, 1200, 1600, 2000, 2400, 2800 and 3397 µg/mL (10% relative survival at 3397 µg/mL) Experiment 2 In the absence of S9 mix: 10, 20, 40, 60, 80, 120, 160, 200 and 250 µg/mL (cytotoxicity: 17% relative survival at 250 µg/mL) In the presence of S9 mix: 150, 300, 600, 1600, 2000, 2400, 2700, 3000 and 3397 µg/mL (16% relative survival at 3397 µg/mL) Experiment 3 In the absence of S9 mix: 15, 30, 90 and 120 µg/mL (cytotoxicity: 11% relative survival at 120 µg/mL)
Treatment:	Experiments I and II : 3 h treatment both without and with S9-mix; Expression period 7 days and a selection period of 11 days.
	Experiment III: 3 h treatment without S9-mix; Expression period 7 days and a selection period of 11 days.
GLP:	in compliance
Study period:	July 2012 – April 2013

Test Procedure
The test item Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) was evaluated in three independent experiments using duplicate cultures each (single cultures for positive controls). The three experiments used a pulse (3-hour), two conducted in the absence and presence of metabolic activation by an Aroclor 1254 induced rat liver post-mitochondrial fraction (S-9) and a third, confirmatory experiment (conducted in the absence of S-9 only) to clarify the data obtained in Experiments 1 and 2. The test material was formulated in purified water.

The ingredient Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) was tested at a broad range of concentrations. The concentrations were selected on the basis of cytotoxicity criteria for final test statistics as follows:
Known mutagens in the presence (Benzo(a)pyrene, BP) or absence of S9 mix (4-nitroquinoline 1-oxide, NQO) were tested at two different concentrations and served as positive controls. Negative controls consisted of cultures treated with the solvent alone (purified water).

Results
Mutation frequencies in solvent negative controls fell within normal ranges, and treatment with positive controls NQO and BP yielded distinct increases in mutant frequency. Accordingly, the study was considered to be valid.

In the absence of S-9 in Experiment 1, no significant increases in mutant frequency (MF) were observed at any concentration analysed (up to a maximum of 200 µg/mL, limited by toxicity) with no linear trend of MF. In Experiment 2, a statistically significant increase in MF was observed only in the absence of S-9 at the highest cytotoxic concentration (250 µg/mL) with a significant linear trend. This isolated increase in Experiment 2 in the absence of S-9 was not reproduced in Experiments 1 and 3 and is therefore considered of no biological relevance.

Also in the presence of metabolic activation when tested up to the limit of cytotoxicity or solubility, there were no relevant statistically significant increases in mutant frequency and no significant linear trends following treatment with Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166).

Conclusion
Under the conditions of this study, Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) was considered not to be mutagenic in the mouse lymphoma assay (*hprt* locus), neither in the absence nor presence of metabolic activation.

Ref: Loyd M (2013)

SCCS comments
In experiment 2, there was a statistically significant increase in mutant frequency with a linear trend up to a concentration of 250 µg/mL without S9 mix, and a statistically significant increase in mutant frequency without linear trend with S9 mix. In the confirmation experiment 3, there was no treatment in the presence of S9-mix. Results from the treatment in the absence of S9-mix do not meet the acceptance criteria, since only 3 concentrations were selected for
the assessment of the study (up to 120 µg/mL). The Applicant has claimed that this is due to high toxicity. However, the concentration of 175 µg/mL was still within the acceptable limits of cytotoxicity (around 10%) and the SCCS is of opinion that it should have been included into the evaluation.

***In vitro* Micronucleus Test in human lymphocytes**

Guideline:	In compliance with the OECD draft guideline OECD 487 (2009)
Test system:	human peripheral blood lymphocytes from two female volunteers.
Replicates:	duplicate cultures in a single experiments with 2 exposure times
Test substance:	Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166)
Batch:	R0027494A 008 L 002
Purity:	~99.9% pure
Solvent:	water
Concentrations:	500, 1500, 2500 and 3397 µg/mL (equivalent to 10 mM) in 3h-treatment in the absence of S9-mix 500, 1500, 2500 and 3397 µg/mL (equivalent to 10 mM) in 3h-treatment in the presence of S9-mix 3, 9, 18, and 30 µg/mL in 24h-treatment in the absence of S9 (limited by cytotoxicity)
Treatment	48 h PHA (phytohaemagglutinin), 3 h treatment and 21 h recovery without or with S9-mix 24 h PHA, 24 h treatment without S9-mix
GLP:	in compliance
Study period:	August 2012 – November 2012

Test Procedure
The test item Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) was evaluated in the absence and presence of metabolic activation (S9 mix prepared from the livers of Aroclor 1254-treated rats).

Duplicate cultures were treated with each concentration of Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) or with known clastogens in the presence (cyclophosphamide, CPA) or absence of S9 (mitomycin C, MMC and vinblastine, VIN). Solvent-treated cultures (purified water, four replicates) were used as negative controls. Cytochalasin B was added after the 3-hour treatments or before the 24-hour treatments The test article was formulated in water for irrigation (purified water). Cytotoxicity was determined in a preliminary cytotoxicity Range-Finder Experiment. The highest concentrations tested was 3397 µg/mL for the 3+21 hour treatments in the absence and presence of S-9 (equivalent to 10 mM) and 80 µg/mL for the 24+0 hour treatment in the absence of S-9 (limited by toxicity).

Results
When compared to concurrent solvent controls, treatment of cultures with positive controls CPA, MMC and VIN resulted in consistent significant increases in MNBN frequencies, thus validating the sensitivity of the test system and procedure used.
Treatment of cells Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) in the absence and presence of S9-mix resulted in frequencies of MNBN cells, which were similar to those observed in concurrent vehicle controls for all concentrations analyzed under all treatment conditions.

Conclusion
Under the conditions of the study, Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) did not produce micronuclei in cultured human peripheral blood lymphocytes neither in the absence nor presence of metabolic activation and was therefore considered to have no clastogenic or aneugenic potential.

Ref: Watters G (2012)

SCCS overall comments
The genotoxic potential of Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) was evaluated in the required battery of *in vitro* studies (Submission I, July 2017) including: the bacterial reverse mutation test, a mammalian cell gene mutation test in mouse lymphoma cells (*Hprt* locus) and a micronucleus assay in cultured human lymphocytes.
The bacterial gene mutation test was negative. However, bacteriotoxicity was noted mainly in the strain TA1537 in the absence of S9-mix even in the lowest concentrations.
Although the study on the gene mutation in mammalian cells (mouse lymphoma cells, *Hprt* locus) raised some concern, the SCCS considered it negative. In the micronucleus test *in vitro*, Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) did not produce micronuclei in cultured human peripheral blood lymphocytes either in the absence or in the presence of metabolic activation and it was therefore considered to have no clastogenic or aneugenic potential.
Following the Applicant's submission of a safety dossier on the new hair dye ingredient Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) in July 2017, several information exchanges occurred between SCCS and the Applicant, mainly pertaining to the genetic toxicity profile of this new hair dye ingredient.
Following a request from the SCCS, the Applicant conducted a complementary bacterial reverse mutation assay (Ames test) on strain TA 1537. The results of this new study confirmed bacteriotoxicity of Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) in S. typhimurium TA1537 strain but only at the highest tested concentration of 5000 µg/plate.
Based on the analysis of all the studies submitted, the SCCS considered Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) as not inducing gene mutations in bacteria and mammalian cells or clastogenic/aneugenic effects in mammalian cells in tested conditions.

3.3.1.2 Mutagenicity / genotoxicity *in vivo*

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3.3.8. Carcinogenicity

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3.3.9 Photo-induced toxicity

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3.3.10. Human data

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3.3.11. Special investigations

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3.3.12. Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

(on-head concentrations of up to 2%)

Absorption through the skin	A	=	1.89 µg/cm²
Skin Area surface	SAS	=	580 cm^2
Dermal absorption per treatment	SAS x A x 0.001	=	1.10 mg
Typical body weight of human	bw	=	60 kg
Systemic exposure dose (SED)	SAS x A x 0.001/bw	=	0.0183mg/kg
No observed adverse effect level (developmental toxicity, oral, rat)	$NOAEL_{sys}$	=	100 mg/kg bw/d
Bioavailability 50%*		=	50 mg/kg bw/d
Margin of Safety	**$NOAEL_{sys}$/SED**	**=**	**2732**

* standard procedure according to the SCCS's Notes of Guidance for the testing of cosmetic ingredients and their safety evaluation.

3.3.13. Discussion

Physicochemical properties

Impurities
Physicochemical data indicate the presence of six impurities and five residual solvents.

Considering MoS calculation based on added information provided by Applicant during the public consultation period (i.e. NOAEL value of 200 mg/kg/day (NOAEL obtained in the 28-day oral toxicity study in rats – OECD 407 (1999)), SCCS agrees on the fact tha the presence of the impurity 3-aminopropyl imidazole at a maximum level of 0.1% in Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) presents no or a negligible risk to human health.

Considering complementary information provided by Applicant during the public consultation period, the exposure to the impurity chloroethane to be considered is equal to 100 microgram per day which is below the NSRL of 150 microgram/day. In addition, such an exposure of 100 microgram / day is far below the virtual safe dose (996 microgram/day) calculated based on the LTD10 with a MoE of 10000. SCCS agrees that the presence of the

impurity chloroethane at a maximum level of 0.05% in Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) presents a negligible risk to human health.

The impurity, 4-aminophenylamine dihydrochloride (synonym p-phenylenediamine dihydrochloride), has already been the subject of an SCCS Opinion (SCCS/1443/11 Revision of 18 September 2012), which raised concerns over its strong skin sensitising potency.

Stability
The information provided on stability of A166 is not convincing. The use of ascorbic acid and argon for the shaking flask method, and the storage conditions (4 ± 4 °C, in the dark under argon), indicates that the test substance is easily oxidised. The Applicant has provided further information on the stability studies performed for the dose formulation used for toxicity studies, where the dissolution media is distilled water. However, details on the solvent media and the procedure used for preparation of solutions are not fully described in the report.

It is necessary to provide data on the stability of A166 under conditions of use with a selective analytical method. Liquid scintillation counting is not a selective method for this purpose. The stability study was performed before mixing the test substance with the developer, whereas it should be performed after the mixing with the developer. Any degradation products should also be chemically characterised.

Toxicological Evaluation

Acute toxicity
The acute oral toxicity of Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) was evaluated following single administration to fasted rats at 500 mg/kg. Animals were observed until day 8 instead of for 14 days, but since no mortality was registered, it can be deduced that the oral LD50 is higher than 500 mg/kg b.w.

Irritation and corrosivity
Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) at the concentration of 10%(w/w) in water applied on reconstructed human epidermis model is considered to be non-irritant to the skin.
A mild to moderate eye irritation potential of the test item cannot be excluded.

Skin sensitisation
Based on the EC3 value of 1.23% observed in Local Lymph Node Assay (LLNA) in mice, Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) is considered as a strong skin sensitiser.

Toxicokinetics
The percutaneous absorption of 2% Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) was investigated *in vitro* in a oxidative hair dye formulation, in the presence of hydrogen peroxide by using human skin preparations. The mean + 1 SD of dermal absorption value, i.e. 1.89 µg/cm^2 (0.48%), will be used for MoS calculation.
A moderate permeability observed in this study supports the default value of 50% for oral bioavailability, which will be used for the calculation of the MoS.

Repeated dose toxicity
Based on limited adverse effects (changes in clinical chemistry and haematology at the highest dose) observed after Repeated dose 90-day oral toxicity study of Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) in wistar rats, the SCCS derives a NOAEL of 300 mg/kg bw/d.

Reproductive toxicity
Methylimidazoliumpropyl phenylenediamine, Cl, 2HCl (A166) is not teratogenic in rats. NOAEL for developmental toxicity is 1000 mg/kg b.w.
NOAEL for maternal toxicity is 100 mg/kg b.w. due to changes in lethargy at 300 and 1000 mg/kg b.w. and weakness, hypoaesthesia and sonnolence and decreased mean body weight gain and mean percent body weight and of absolute uterine weight and uterine weight corrected to body weight at 1000 mg/kg b.w.

Mutagenicity / genotoxicity
Based on the analysis of all the *in vitro* studies submitted, the SCCS considered Methylimidazoliumpropyl p-phenylenediamine, Cl, 2HCl (A166) as not inducing gene mutations in bacteria and mammalian cells or clastogenic/aneugenic effects in mammalian cells in tested conditions.

4. CONCLUSION

1. In light of the data provided, does the SCCS consider Methylimidazoliumpropyl p-phenylenediamine HCl (A166), safe when used in oxidative hair colouring products up to a maximum on-head concentration of 2%?

Based on the full set of informations provided by Applicant (including added information provided by Applicant during the commenting period), the SCCS considers Methylimidazoliumpropyl p-phenylenediamine HCl (A166), 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 Methylimidazoliumpropyl p-phenylenediamine HCl (A166) in cosmetic products?

The SCCS has noted that Methylimidazoliumpropyl p-phenylenediamine HCl (A166) is a strong skin sensitiser.

5. MINORITY OPINION

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7. GLOSSARY OF TERMS

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

8. LIST OF ABBREVIATIONS

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