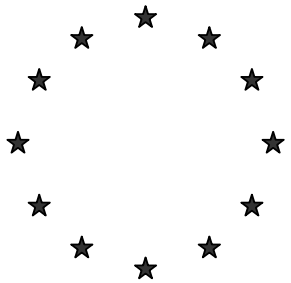
COMPETENT AUTHORITY REPORT



1,2-Benzisothiazol-3-(2*H*)-one (BIT) (PT 13)

Document III-A

Active Substance

Rapporteur Member State: Spain
November 2021

Section A1 Applicant

Annex Point IIA, I 1

			Official use only
1.1	**Applicant**	Name: **Lonza Cologne GmbH** Contact person: [redacted] --- Name: **Thor GmbH** [redacted] --- Name: **Laboratorios Miret S.A.** [redacted]	
1.2	**Manufacturer of Active Substance** (if different)	Information relating to Registrants manufacturing sites and third party suppliers are commercially sensitive and considered Confidential. Full details are given in the Confidential Section.	X1
1.3	**Manufacturer of Product(s)** (if different)	Please, see Confidential Doc IIIA	X1

Evaluation by Competent Authorities

EVALUATION BY RAPPORTEUR MEMBER STATE

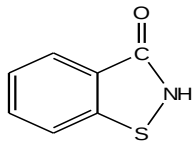
Section A1 Applicant

Annex Point IIA, I 1

Date	*December 2007* *October 2020* *August 2021*
Conclusion	*The applicant's version is adopted with clarifications.*
Remarks	*No further remarks* [redacted] *(X1) this information is included in the Doc IIIA confidential.*

Section A2 Identity

Annex point IIA, II 2 **Identity of Active Substance**

Subsection (Annex Point)			Official use only
2.1	**Common name (IIA, II)**	BIT 1,2 Benzisothiazolin-2-one 1,2 Benzisothiazol-3-(2*H*)-one	
2.2	**Chemical name (IIA, II 2.2)**	Benzo-[*d*]-isothiazol-2-one	
2.3	**Manufacturer´s development code number(s) (IIA, II 2.3)**	Refer to TNG Justification for Non-submission of Data IIIA 2.3.	
2.4	**CAS No and EC numbers (IIA, II 2.4)**		
2.4.1	CAS-No	2634-33-5	
2.4.2	EC-No	220-120-9 (EINECS)	
2.4.3	Other	Not applicable	
2.5	**Molecular and structural formula, molecular mass (IIA, II 2.5)**		
2.5.1	Molecular formula	C_7H_5NOS	
2.5.2	Structural formula		
2.5.3	Molecular mass	151.19 g/mol	
2.6	**Method of manufacture of the active substance (IIA, II 2.6)**	The manufacture of BIT consists of a multi-stage process, which is Confidential and specific for each of the Active Substance suppliers. Full details are provided in the Confidential Section of the dossier.	**X1**

Section A2 Identity

Annex point IIA, II 2 **Identity of Active Substance**

2.7	**Specification of the purity of the active substance, as appropriate (IIA, II 2.7)**	**g/kg (as Organic purity)** TGAS: > 946 Individual source Specification is supplied in the Confidential Section	**% w/w (as Organic purity)** TGAS: > 94.6 Individual source Specification is supplied in the Confidential Section	**X2** **X3**
2.8	**Identity of impurities and additives, as appropriate (IIA, II 2.8)**	See separate standard format in the Confidential Section		**X1**
2.8.1	Isomeric composition	Not relevant		
2.9	**The origin of the natural active substance or the precursor(s) of the active substance (IIA, II 2.9)**	The precursor(s) of the active substance are sourced from mainstream basic chemicals suppliers.		

Evaluation by Competent Authorities

EVALUATION BY RAPPORTEUR MEMBER STATE

Date

December 2007

May 2020.

October 2020.

August 2021

Conclusion

The applicant's version is adopted with clarifications.

Section A2 Identity

Annex point IIA, II 2 **Identity of Active Substance**

Remarks

No further remarks

(X1) This information is included in the Doc IIIA confidential.

[redacted]

[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]

[redacted]

Section A2.10
Annex Point IIA2.10

Exposure data in conformity with Annex VIIA to Council Directive 92/32/EEC (OJ No L, 05.06.1992, p. 1) amending Council Directive 67/548/EEC

Subsection		Official use only
2.10.1	**Human exposure towards active substance**	
2.10.1.1 Production i) Description of process ii) Workplace description iii) Inhalation exposure iv) Dermal exposure	No risk assessment has been made in this document for operators manufacturing the active substance or for operators involved in production of the biocidal product. Operator exposure at this level is considered under the requirements of The Chemical Agents at Work Directive (98/24/EEC, within Directive 89/391/EEC), and controlled using engineering controls and PPE and RPE as appropriate, according to The Personal Protective Equipment at Work Regulations 1992 (EU Directive 89/656/EEC). These Regulations competently control for operator exposure to the biocides and substances of concern in a formulation. As it is not the intention of the BPD to overlap existing legislation, it is not considered necessary to undertake assessments of production and/or formulation in this submission.	
2.10.1.2 Intended use(s)		
1. Professional users (primary exposure)		**X**
i) Description of application process	The following exposure scenarios need to be addressed; Scenario 1: (Primary exposure) <u>Addition of 20% BIT/GLYCOL during manufacture of MWF Concentrate</u> The method of application is by incorporation of the biocide into the coolant concentrate at the time of manufacture. Metalworking fluid concentrates are made by batch process in enclosed mixing vessels. The biocide charging operation is assumed to be carried out once per day by pumped transfer or, in cases of very small batches, by manual pouring. Manual pouring is expected to occur rarely, if at all, because the MWF products are generally manufactured on a large scale. Typical batch sizes for metalworking fluid concentrates could range from 2200 kg to 11000 kg. Therefore, for a mid range 5000 kg batch, a worker would have to charge approximately 150 litres of 20% BIT/GLYCOL to the mixing vessel. All users are industrial operatives and would be expected to wear the PPE stipulated on the 20% BIT/GLYCOL label. Scenario 2: (Primary exposure) <u>Tankside Addition of 20% BIT/GLYCOL to prepared MWF</u> The need for a “top up” dose using a tankside biocide would be based	

Section A2.10
Annex Point IIA2.10

Exposure data in conformity with Annex VIIA to Council Directive 92/32/EEC (OJ No L, 05.06.1992, p. 1) amending Council Directive 67/548/EEC

on a microbiological evaluation of the coolant in the system.

The biocide is added to the system "sump" by pumping or manual pouring, depending upon the system size (volume) and recommended biocide dose. System sizes vary from 100-200 litres for stand alone machines with individual sumps to ca. 40,000 or 100,000 litres for large central systems which feed many machines.

The amount of biocide added into a system is based on the recommended dose and the total volume of coolant. For example if the system size is 10,000 litres and the recommended biocide dose is 300 ppm (0.03%) then 15 litres of 20% BIT/GLYCOL would be required to treat the system. From the ESD for PT13, it is expected that:

fluid dilution and/or biocide addition =1 per week, 10 minutes.

All users are either industrial operatives or professional workers and would be expected to wear the PPE stipulated on the 20% BIT/GLYCOL label.

Scenario 3: (Secondary exposure)

Mixing/loading of treated MWF concentrate to produce working strength MWF

The MWF concentrate is added to the system "sump" by pumping or manual pouring, depending on the system size (volume) and recommended dilution. System sizes vary from 100-200 litres for stand alone machines with individual sumps to ca. 40,000 or 100,000 litres for large central systems which feed many machines.

The expected concentration of BIT in the MWF concentrate is up to 0.6% w/w (6000 ppm) as BIT. Typical dilution of the concentrate is 1:20 and even a small stand alone system would require 5 to 10 litres of concentrate. A large scale operation with a 10,000 litre sump would require 500 litres of concentrate. From the ESD for PT13, it is expected that

fluid dilution and/or biocide addition = 1 per week, 10 minutes

All users are either industrial operatives or professional workers and would be expected to wear the PPE stipulated by the label of the MWF concentrate.

Scenario 4: (Secondary exposure)

Machining operations using prepared MWF

Workers performing machining operations may be exposed to MWF during such tasks.

The expected concentration of BIT in the prepared MWF is up to 0.03% w/w (300 ppm) as BIT (=0.15% (1500 ppm) 20% BIT/GLYCOL).

All users are either industrial operatives or professional workers and would be expected to wear the PPE demanded by the hazardous nature of the machining tasks being undertaken or metal pieces being

Section A2.10 Exposure data in conformity with Annex VIIA to Council Directive 92/32/EEC (OJ No L, 05.06.1992, p. 1) amending Council Directive 67/548/EEC

Annex Point IIA2.10

handled. The following operations are expected:

tool setting and dismantling (4 per day, 10 minutes per event)

The worker may be exposed to metalworking fluid containing 300 ppm BIT when they are in contact with shaped metal, which could be covered with a thin film of metal working fluid. The worker would always wear gloves during contact with the shaped metal.

metalworking - operator near to machine (1 hour per day)

Scenarios 1, 2 and 3 all involve mixing and loading. The route of exposure relevant to mixing/loading is dermal contamination. The ingestion route is not considered relevant for professional users. Inhalation is not considered relevant because there are no high shear operations to generate an aerosol and the active substance is not volatile.

The nature of the MWF (oil or water based) is not relevant to mixing/loading operations but it is relevant to inhalation exposure in Scenario 4.

ii) Workplace description

Metalworking fluids are used exclusively by professional users in industrial, controlled environments. Because of the nature of many of the components in metalworking fluids, exposure is tightly controlled with published guidance for industry. An example of the guidance is provided by the UK HSE (http://www.hse.gov.uk/metalworking/ecoshh.htm) which provides guidance on minimising exposure at every stage of the use process.

iii) Inhalation exposure

Exposure scenario	PPE	Inhalation
Scenario 1 & 2 (using same model and use pattern) Mixing/Loading 20% BIT/GLYCOL	Tier 1: None	1.39E-03
	Tier 2: Protective Clothing	1.39E-03
Scenario 3 Mixing/Loading MWF Concentrate	Tier 1: None	4.17E-05
	Tier 2: Protective Clothing	4.17E-05
Scenario 4 Machining operations	Tier 1: None	1.15E-05
	Tier 2: Gloves	1.15E-05

iv) Dermal exposure

Exposure scenario	PPE	Dermal

Section A2.10 Exposure data in conformity with Annex VIIA to Council Directive 92/32/EEC (OJ No L, 05.06.1992, p. 1) amending Council Directive 67/548/EEC

Annex Point IIA2.10

Scenario 1 & 2 (using same model and use pattern) Mixing/Loading 20% BIT/GLYCOL	Tier 1: None	1.81E-03
	Tier 2: Protective Clothing	3.61E-04
Scenario 3 Mixing/Loading MWF Concentrate	Tier 1: None	5.42E-05
	Tier 2: Protective Clothing	1.08E-05
Scenario 4 Machining operations	Tier 1: None	2.76E-02
	Tier 2: Gloves	1.38E-03

2. Non-professional users including the general public — X4

(i) via inhalational contact — This application is for professional use only.

(ii) via skin contact — This application is for professional use only.

(iii) via drinking water — This application is for professional use only.

(iv) via food — This application is for professional use only.

(v) indirect via environment — This application is for professional use only.

2.10.2 Environmental exposure towards active substance

2.10.2.1 Production

(i) Releases into water

(ii) Releases into air

(iii) Waste disposal

No risk assessment has been made in this document for environmental exposure during manufacturing the active substance or for in production of the biocidal product. Environmental exposure at this level is regulated under the IPPC Directive. As it is not the intention of the BPD to overlap existing legislation, it is not considered necessary to undertake assessments of production and/or formulation in this submission.

Section A2.10
Annex Point IIA2.10

Exposure data in conformity with Annex VIIA to Council Directive 92/32/EEC (OJ No L, 05.06.1992, p. 1) amending Council Directive 67/548/EEC

2.10.2.2 Intended use(s)

Affected compartment(s):

water	99.5%
sediment	9.8×10^{-3}%
air	1.51×10^{-4}%
soil	0.442%

Predicted concentration in the affected compartment(s)

water — Emulsifiable MWF

Compartment	Values Industrial users	Values Professional users
PEC for micro-organisms in the STP (mg/L)	0.0219	5.48E-03
Local PEC in surface water during emission episode (dissolved) (mg/L)	2.19E-03	5.48E-04
Local PEC in fresh-water sediment during emission episode (mg/kg wwt)	0.0207	5.18E-03
Local PEC in groundwater under agricultural soil (mg/L)	2.54E-06	6.36E-07

Water Soluble MWF

Compartment	Value
PEC for micro-organisms in the STP (mg/L)	1.14E-03
Local PEC in surface water during emission episode (dissolved) (mg/L)	1.14E-04
Local PEC in fresh-water sediment during emission episode (mg/kg wwt)	1.08E-03

Section A2.10 Exposure data in conformity with Annex VIIA to Council Directive 92/32/EEC (OJ No L, 05.06.1992, p. 1) amending Council Directive 67/548/EEC

Annex Point IIA2.10

Local PEC in groundwater under agricultural soil (mg/L)	1.33E-07

sediment

Emulsifiable MWF

Compartment	Values Industrial users	Values Professional users
Local PEC in fresh-water sediment during emission episode (mg/kg wwt)	0.0207	5.18E-03

Water Soluble MWF

Compartment	Value
Local PEC in fresh-water sediment during emission episode (mg/kg wwt)	1.08E-03

air

Not considered due to properties of BIT

soil

Emulsifiable MWF

Outputs	Values Industrial users	Values Professional users
Local PEC in agricultural soil (total) averaged over 30 days (mg/kg wwt)	1.09E-04	2.73E-05
Local PEC in agricultural soil (total) averaged over 180 days (mg/kg wwt)	1.82E-05	4.55E-06

Water Soluble MWF

Outputs	Values
Local PEC in agricultural soil (total) averaged over 30 days (mg/kg wwt)	5.7E-06
Local PEC in agricultural soil (total) averaged over 180 days (mg/kg wwt)	9.5E-07

Evaluation by Competent Authorities

EVALUATION BY RAPPORTEUR MEMBER STATE

Section A2.10
Annex Point IIA2.10

Exposure data in conformity with Annex VIIA to Council Directive 92/32/EEC (OJ No L, 05.06.1992, p. 1) amending Council Directive 67/548/EEC

Date	*March 2010* *March 2015*
Materials and method	
Conclusion	
Reliability	
Acceptability	*Applicant's version is adopted with modifications.*
Remarks	***X*** *The specific use being supported is the incorporation of BIT into a Metal Working Fluid (MWF) concentrate or as a tank-side treatment. 20%BIT/Glycol is either used by the manufacturer of the MWF concentrate and diluted at a maximum of 0.6% BIT in the concentrate (6000 ppm a.i.), which is then diluted in the end MWF fluid up to a maximum of 0.03% BIT (300 ppm a.i.), or it can be directly used as it by the end-user and is incorporated in the MWF to get the same BIT concentration in it resulting in 0.03% BIT maximum.* *The exposure to BIT was calculated based in the selected models and default values from TNsG on Human Exposure, 2002, taking into account the User Guidance to report 2002 and the HEEG opinion on Human exposure assessment to biocidal products used in metalworking fluids (PT 13), (Ispra, 22/09/2008).* *See details in Doc. II-B.* March 2015: Human Exposure Assessment will be revised to take into account comments received from MS and applicant after 2012 initial submission as well as ECHA BPWG's agreements.

Section A2	**Identity**	
Subsection A2.3	**MANUFACTURER´S DEVELOPMENT CODE NUMBER(S)**	
Annex Point IIA2.3		
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ]	**Technically not feasible [ ]** **Scientifically unjustified [ ]**	
Limited exposure [ ]	**Other justification [X]**	
Detailed justification:	Manufacturer development code number(s) are not applicable to BIT since this active substance is not in the development phase.	
Undertaking of intended data submission [ ]		
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*December 2007.*	
Evaluation of applicant's justification	*The non-submission of data is justified*	
Conclusion	*Acceptable*	
Remarks	*No further remarks*	

Section A3 Physical and Chemical Properties

Annex point IIA, III 3 **PHYSICAL AND CHEMICAL PROPERTIES OF ACTIVE SUBSTANCE**

Subsection (Annex point)	Method	Purity / Specification	Results	Remarks / Justification	GLP (Y/N)	Reliability	Reference	Official use only
3.1 Melting point, boiling point, relative density (IIA, III 3.1)								
3.1.1 Melting point	EC A.1	Purity = 99.8%	Melting Point = 157.1 ± 0.4 °C at atmospheric pressure	The melting point was determined using DSC by heating from 20 °C to 180 °C with a ramp of 10°C/min. Two determinations were made and the results were averaged.	Y	1	[redacted] 2002; Physical Chemical tests on pure BIT. [redacted] Project 1274163	
	EC A.1 OECD 102 EPA OPPTS 830.7200	Technical grade active substance (Purity = 73.6%)	The melting point of BIT was determined to be 156.6 ± 0.1 °C.	Determined using DSC. The main test was performed at a rate of 5 K/min and the results from two tests were averaged (156.5 and 156.6°C).	Y	1	[redacted] 1998; Determination of the Melting Point/ Melting Range of [redacted] Study Project No. 702731	
3.1.2 Boiling point	EC A.2	Purity = 99.8%	No boiling point could be determined since the sample was observed to have decomposed. The	DSC was used to determine the boiling point. The sample was heated from 20 °C to	Y	1	[redacted] 2002; Physical Chemical tests on pure BIT.	

Section A3 Physical and Chemical Properties

Annex point IIA, III 3 **PHYSICAL AND CHEMICAL PROPERTIES OF ACTIVE SUBSTANCE**

Subsection (Annex point)	Method	Purity / Specification	Results	Remarks / Justification	GLP (Y/N)	Reliability	Reference	Official use only
			DSC trace shows that the sample decomposed at a temperature above 300 ºC.	450 ºC at a rate of 20 ºC/min.			[redacted] Project 1274163	
	EC A.2 OECD 103 EPA OPPTS 830.7220	Purity = > 99%	The boiling point of BIT was determined to be 328.7 °C.	Determined using DSC. The main test performed at a rate of 5 K/min - two tests were performed (250-400 °C and 200-400 °C). The results from the two tests (328.5 and 328.9°C) were averaged.	Y	1	[redacted] 2002; Determination of the Boiling Point/ Boiling Range of 1,2 Benzisothiazol-3-(2*H*)-one. [redacted] Study Project No. 840980	
3.1.3 Bulk density/ relative density	EC A.3 OECD 109 EPA OPPTS 830.7300	Purity = > 99%	Relative Density = 1.483 at 20 °C.	Three determinations were made and the results were averaged. The relative standard deviation was 0.07%.	Y	1	[redacted] 2002; Determination of the Density of 1,2-benzisothiazol-3-(2*H*)-one (BIT). Study No. RS/01/025	**X1**
	EC A.3	Purity = > 99%	Relative Density = 1.44 at 20 °C.	Three determinations were made and the results were averaged. The relative standard deviation was 0.1%.	Y	1	[redacted] 2007; Determination of the Relative Density of [redacted] BIT. Report	**X2**

Section A3 Physical and Chemical Properties

Annex point IIA, III 3 **PHYSICAL AND CHEMICAL PROPERTIES OF ACTIVE SUBSTANCE**

Subsection (Annex point)	Method	Purity / Specification	Results	Remarks / Justification	GLP (Y/N)	Reliability	Reference	Official use only
							No. B 046/2006.	
3.2 Vapour pressure and Henry`s Law Constant (IIA, III 3.2)								
Vapour pressure	EC A.4	Three different BIT samples were used to produce a linear plot Sample 1 BIT (Purity = > 99%) Sample 2 'Polaroid Quality BIT' (Purity =	Vapour Pressure at 20 °C: 6.3×10^{-5} Pa (4.7×10^{-7} mmHg) Vapour Pressure at 25 °C: 1.4×10^{-4} Pa (1.1×10^{-6} mmHg) Vapour Pressure at 30 °C: 3.1×10^{-4} Pa (2.3×10^{-6} mmHg)	Values for vapour pressure of BIT at 20 °C, 25 °C and 30 °C were extrapolated from a linear plot of vapour pressure against temperature. The vapour pressure data was derived from 8 determinations (a total of eight determinations were made using three different BIT analytical standards) over the temperature range of 64.2 °C to 135.6 °C.	Y(N)[1]	1	[redacted] 2000; Determination of the Vapour Pressure and Atmospheric Concentration of Benzisothiazolone. [redacted] No. 00036	**X3**

[1] Only determinations made using sample 3 (series 3) were performed to GLP (GLP Report No 175, Williams). Sample sets 1 and 2 have been included to reinforce the data. The three sets are in close agreement and therefore the plot shows good linearity]

Section A3 Physical and Chemical Properties

Annex point IIA, III 3 **PHYSICAL AND CHEMICAL PROPERTIES OF ACTIVE SUBSTANCE**

Subsection (Annex point)	Method	Purity / Specification	Results	Remarks / Justification	GLP (Y/N)	Reliability	Reference	Official use only
		99.9%) Sample 3 BIT (Purity = 99.5%)						
	EC A.4 OECD 104 EPA OPPTS 830.7950	Technical grade active substance (Purity = 73.6%)	The vapour pressure of BIT at 20°C and 25 °C were extrapolated by linear regression: 1.8 x 10^{-4} Pa at 20 °C* 3.7 x 10^{-4} Pa at 25 °C.	Due to the low vapour pressure of the test substance the tests using the gas saturation method had to be performed at 60, 70 and 80 °C. *Vapour pressure at 20°C was calculated by the notifier (extrapolated from the linear regression). The calculation is presented in Table 3.2-2.	Y	1	[redacted] 1998; Determination of the Vapour Pressure of [redacted] Study No. 702696	**X4**
Henry´s Law Constant	Described in: Pesticides Research, 64 – Technical Documentation of PestSurf, a Model describing Fate and Transport of Pesticides in	Calculated, based upon VP, and WSol derived using > 99% BIT	Calculated result: HENRY = 7.40× 10^{-6} Pa.m^3/mol at 20°C	Calculated from the vapour pressure (20°C) and solubility (20°C, pH 6.7) reported in Sections 3.2 and 3.5, respectively. The calculation is presented in Table 3.2.1-1.	Y	1	EPI Suite ™Version 3.1.2	

Section A3 Physical and Chemical Properties

Annex point IIA, III 3 **PHYSICAL AND CHEMICAL PROPERTIES OF ACTIVE SUBSTANCE**

Subsection (Annex point)	Method	Purity / Specification	Results	Remarks / Justification	GLP (Y/N)	Reliability	Reference	Official use only
	Surface Water for Danish Conditions							
3.3 Appearance (IIA, III 3.3)								
3.3.1 Physical state 1	Visual Assessment.	Purity = 99.8%	Solid powder at ambient temperature.	-	Y	1	[redacted] 2002; Physical Chemical tests on Pure BIT. [redacted] Project 1274163	
3.3.1 Physical state 2	Visual Assessment	Technical grade active substance (Purity = 73.2%)	Damp powder at ambient temperature.	-	Y	1	[redacted].; 2000; BIT Water Solubility at pH 5, 7 and 9. [redacted] Project 1248276	
3.3.2 Colour 1	Visual Assessment.	Purity = 99.8%	White at ambient temperature.	-	Y	1	[redacted] 2002; Physical Chemical	

Section A3 Physical and Chemical Properties

Annex point IIA, III 3 **PHYSICAL AND CHEMICAL PROPERTIES OF ACTIVE SUBSTANCE**

Subsection (Annex point)	Method	Purity / Specification	Results	Remarks / Justification	GLP (Y/N)	Reliability	Reference	Official use only
							tests on pure BIT. [redacted] Project 1274163	
3.3.2 Colour 2	Visual Assessment	Technical grade active substance (Purity = 73.2%)	Brown at ambient temperature.	-	Y	1	[redacted].; 2000; BIT Water Solubility at pH 5, 7 and 9. [redacted] Project 1248276	
3.3.3 Odour 1	None	Pre-dried technical grade active substance (Purity = 94.2%)	No obvious odour.	-	Y	2	[redacted] 1991; Physical/ Chemical Characteristics of [redacted] Project 175	
3.4 Absorption spectra (IIA, III 3.4)								
UV/VIS	OECD 101	Purity = > 99%	The molar absorption coefficients for BIT with a purity of > 99% in HPLC water, NaOH (0.1 mol/L), HCl (0.1 mol/L) are	The test was conducted with concentrations of 12.5 and 25 mg/L in HPLC water, NaOH (0.l mol/L) and HCL (0.l mol/L) and methanol. The	Y	I	[redacted] 2004; BIT-Standard: UV-VIS Absorption Spectra (Spectrophotometric Method). [redacted]	

Section A3 Physical and Chemical Properties

Annex point IIA, III 3 **PHYSICAL AND CHEMICAL PROPERTIES OF ACTIVE SUBSTANCE**

Subsection (Annex point)	Method	Purity / Specification	Results	Remarks / Justification	GLP (Y/N)	Reliability	Reference	Official use only
			summarised in Table A.3.4.1-1.	wavelength range was 200 - 750 nm. Scans for absorbance maxima and detailed scans of the detected absorption maxima were performed. The molar absorption coefficients were calculated for each test condition and maxima capable of being resolved.			[redacted] Project No. 040913TW, Study No. CPA97651N	
IR	None	Purity = >99%	The IR-spectra obtained shows characteristic absorption bands in the range between 4000 and 400 cm^{-1}. The data are presented in Table A3.4.2-1 and Figure A3.4.2-1.	The infrared spectrum was obtained by preparation of a pellet with test item and potassium bromide. The spectrum was recorded on a Perkin Elmer Spectrum BX II Fourier Transform Infrared Spectrometer in the range of 4000 to 400 cm^{-1}.	N	2	[redacted] 2007; Characterization of the Molecular Structure of [redacted] BIT by ^{1}H-NMR- and IR-spectroscopy (non GLP). [redacted]	
NMR	None	Purity = 99.8%	The spectra obtained were consistent with the proposed structure. The results are presented in Table A3.4.3-1 and Figure A3.4.3-1.	The test sample was examined by proton NMR and ^{13}C as a solution in deuterated dimethylsulphoxide (d_6-DMSO) using a Bruker Avance 400 spectrometer.	Y	1	[redacted] 2002; Physical Chemical tests on pure BIT. [redacted] Project 1274163	

Section A3 Physical and Chemical Properties

Annex point IIA, III 3 **PHYSICAL AND CHEMICAL PROPERTIES OF ACTIVE SUBSTANCE**

Subsection (Annex point)	Method	Purity / Specification	Results	Remarks / Justification	GLP (Y/N)	Reliability	Reference	Official use only
	None	Purity = > 99%	All NMR-spectra of the test items corresponds with the proposed structure. The data are presented in Table A3.4.3-2 and Figure A3.4.3-2.	Nuclear Magnetic Resonance spectra were recorded on a Bruker AC 300 E NMR spectrometer (300 MHz for ^{1}H-NMR) with Dimethylsulfoxide-d_6 as solvent. The chemical shifts of the test sample signals were assigned by referencing Tetramethylsilane to 0 ppm.	N	1	[redacted] 2007; Characterization of the Molecular Structure of [redacted]® BIT by 1H-NMR- and IR-spectroscopy (non GLP); [redacted]	
MS	None	Purity = > 98%	Mass spectra are presented in Figure A3.4.4-1. The molecular ion for BIT at 152 m/z ($M^{+\cdot}$+H) was observed	Characterisation of BIT by LC-MS. A 0.25 mg/ml solution was prepared dissolving BIT in DMF. The solution was then made to volume with acetonitrile and analysed by LC-MS[2] with electropray in positive mode.	Y	1	[redacted] 2007; Analysis of Five Representative Batches of [redacted] BIT. [redacted] Report No. B 054/2006; Draft.	

[2] Column: Waters Symmetry Shield RP-8 (250 mm × 4.6 mm, 5 µ)
Mobile phase A: 0.01 mol/L formic acid/Acetonitrile (75/25, v/v)
Mobile Phase B: 0.01 mol/L formic acid /Acetonitrile (20/80, v/v)
A gradient (100% A to 100% B over 22 minutes) with a flow rate of 1.0 mL/min was employed and the injection volume was 20 µL.

Section A3 Physical and Chemical Properties

Annex point IIA, III 3 **PHYSICAL AND CHEMICAL PROPERTIES OF ACTIVE SUBSTANCE**

Subsection (Annex point)	Method	Purity / Specification	Results	Remarks / Justification	GLP (Y/N)	Reliability	Reference	Official use only
3.5 Solubility in water (IIA, III 3.5)								
Water solubility 1	EC A.6	Technical grade active substance (Purity = 73.2%)	Distilled water (pH 5.36) solubility at 20 ± 1 °C = 1153 mg/L. Nominal pH 5(buffered) Mean measured pH 5.07 solubility at 10 ± 1 °C, 20 ± 1 °C, 30 ± 1 °C was 810 mg/L, 1113 mg/L and 1631 mg/L. Nominal pH 7(buffered) Mean measured pH 6.78, solubility at 10 ± 1 °C, 20 ± 1 °C, 30 ± 1 °C was 1301 mg/L, 1707 mg/L and 2438 mg/L. Nominal pH 9 (buffered) Mean measured pH 7.36, 10 ± 1°C, 20 ± 1°C, 30 ± 1°C was 3181 mg/L, 3628 mg/L and 4220 mg/L,	All data presented are derived from the mean result of 4 tests which were conducted over a period of 96 hours. The analysis was performed using HPLC with DAD. The self adjustment in pH observed for the pH 9 buffered solution in this study can also be attributed to the dissociation of BIT.	Y	1	[redacted].; 2000; BIT Water Solubility at pH 5, 7 and 9. [redacted] Project 1248276	**X5**

Section A3 Physical and Chemical Properties

Annex point IIA, III 3 **PHYSICAL AND CHEMICAL PROPERTIES OF ACTIVE SUBSTANCE**

Subsection (Annex point)	Method	Purity / Specification	Results	Remarks / Justification	GLP (Y/N)	Reliability	Reference	Official use only
			respectively.					
Water solubility 2	EC A.6 OECD 105 EPA OPPTS 830.7840	Technical grade active substance (Purity = 73.6%)	The water solubility of BIT was 1.085 g/L with a relative standard deviation of 0.92%.	In the main test duplicate samples were shaken for 24, 48 and 72 hours at 30 °C. The flasks were then equilibrated for 24 hours at 20 °C and BIT was determined by HPLC with UV detection.	Y	1	[redacted] 1998; Determination of the Water Solubility of [redacted] Study Project No. 705431.	**X6**

Section A3 Physical and Chemical Properties

Annex point IIA, III 3 **PHYSICAL AND CHEMICAL PROPERTIES OF ACTIVE SUBSTANCE**

Subsection (Annex point)	Method	Purity / Specification	Results	Remarks / Justification	GLP (Y/N)	Reliability	Reference	Official use only
Water solubility 3	EC A.6 OECD 105 EPA OPPTS 830.7840	Purity = 99%	The water solubility of BIT was 0.727, 0.938, and 1.196 g/L at 10, 20 and 30 °C, respectively (pH 4.8). The water solubility of BIT was 0.938, 1.288, and 1.651 g/L at pH 4.8, 6.7 and 9.1, respectively (20 °C).	The solubility of BIT was determined using a shake flask method. A preliminary test was not performed since data was available on the water solubility of BIT (1.1 g/L).	Y	1	[redacted].; 2002; "Determination of the Water Solubility of 1 ,2-BIT at a Range of Temperatures and pHs. [redacted] Study No. RS/01/029.	
3.6 Dissociation constant (-)								
Dissociation constant (-) 1	EPA OPPTS 830.7370 USP Method 761	Purity = 100%	pK_a of BIT = 7.2 at 25°C and 7.2 pH	The dissociation constant of the test substance was determined by Nuclear Magnetic Resonance Spectra (^{1}H-NMR).	Y	1	[redacted] 2001; [redacted] Dissociation Constant. [redacted] Report No. SSL00801.	
Dissociation constant (-) 2	OECD 112	Purity = 99.5%	pK_a of BIT = 7.5 ± 0.1 at 25 °C	An average value was calculated from determinations made at 25 °C for a 0.007 M aqueous	Y	1	[redacted] 1991; Physical/ Chemical Characteristics of [redacted]	

Section A3 Physical and Chemical Properties

Annex point IIA, III 3 **PHYSICAL AND CHEMICAL PROPERTIES OF ACTIVE SUBSTANCE**

Subsection (Annex point)	Method	Purity / Specification	Results	Remarks / Justification	GLP (Y/N)	Reliability	Reference	Official use only
				saturated solution, 0.005 M solution (5:95, MeOH:H20, v/v) and a 0.01 M solution (5:95, MeOH:H_2O, v/v).			[redacted] Project 175.	
3.7 Solubility in organic solvents, including the effect of temperature on solubility (IIIA, III 1)	None	Pre-dried technical grade active substance (Purity = 94.2%)	67000 mg/L in methanol at 24ºC 13000 mg/L in acetonitrile at 23ºC 42000 mg/L in acetone at 24ºC 31000 mg/L in dichloromethane at 23ºC 5000 mg/L in toluene at 24ºC 23000 mg/L in ethyl acetate at 23ºC 100 mg/L in hexane at 23ºC	The solutions were stirred in conical flasks with stoppers at room temperature for 4 hours. The solutions were then allowed to stand for a minimum of 30 minutes prior to filtering. Filtrates were examined by spectrophotometer to determine the solubility of the test substance. The effect of temperature was not investigated in this study. Refer to Justification for Non-submission of Data (IIIA 3.7_1) for calculated data which demonstrates the effect of temperature on solubility.	Y	1	[redacted].; 1991; Physical/ Chemical Characteristics of [redacted] Project 175.	

Section A3 Physical and Chemical Properties

Annex point IIA, III 3 **PHYSICAL AND CHEMICAL PROPERTIES OF ACTIVE SUBSTANCE**

Subsection (Annex point)	Method	Purity / Specification	Results	Remarks / Justification	GLP (Y/N)	Reliability	Reference	Official use only
3.8 Stability in organic solvents used in b.p. and identity of relevant breakdown products (IIIA, III 2)				Refer to TNG Justification for Non-submission of Data A3.8.				**X7**

Section A3 Physical and Chemical Properties

Annex point IIA, III 3 **PHYSICAL AND CHEMICAL PROPERTIES OF ACTIVE SUBSTANCE**

Subsection (Annex point)	Method	Purity / Specification	Results	Remarks / Justification	GLP (Y/N)	Reliability	Reference	Official use only
3.9 Partition coefficient n-octanol/water (IIA, III 3.6)								

Section A3 Physical and Chemical Properties

Annex point IIA, III 3 **PHYSICAL AND CHEMICAL PROPERTIES OF ACTIVE SUBSTANCE**

Subsection (Annex point)	Method	Purity / Specification	Results	Remarks / Justification	GLP (Y/N)	Reliability	Reference	Official use only
$logP_{ow}$ 1	EC A.8 OECD 117 EPA OPPTS 830.7570	Purity = > 99%	$logP_{ow}$ of BIT at 10, 20 and 30 °C (pH7) = 0.63, 0.70 and 0.76, respectively. $logP_{ow}$ of BIT at pH 5, 7, and 9 (20 °C) = 0.99, 0.70 and -0.90, respectively. The $logP_{ow}$ of BIT shows a significant dependence on pH and is only marginally affected by temperature.	The test material retention times were determined with duplicate samples and the retention time of all materials was determined at 10, 20 and 30 °C (pH 7) and at pH 5, 7 and 9 (20 °C).	Y	1	[redacted]; 2002; Determination of the Partition Coefficient (n-octanol/water) of 1 ,2-BIT at a range of Temperatures and pHs. [redacted] Study No. RS/01/021.	

Section A3 Physical and Chemical Properties

Annex point IIA, III 3 **PHYSICAL AND CHEMICAL PROPERTIES OF ACTIVE SUBSTANCE**

Subsection (Annex point)	Method	Purity / Specification	Results	Remarks / Justification	GLP (Y/N)	Reliability	Reference	Official use only
$logP_{ow}$ 2	Calculation	Not Applicable	$LogP_{ow}$ = 0.64	This estimation supports the measured $logP_{ow}$	N	2	EPI Suite™ Version 3.1.2	**X8**
3.10 Thermal stability, identity of relevant breakdown products (IIA, III 3.7)								

Section A3 Physical and Chemical Properties

Annex point IIA, III 3 **PHYSICAL AND CHEMICAL PROPERTIES OF ACTIVE SUBSTANCE**

Subsection (Annex point)	Method	Purity / Specification	Results	Remarks / Justification	GLP (Y/N)	Reliability	Reference	Official use only
Termal stability 1	DSC	Purity = 99.8%	Thermal Stability up to at least 300ºC.	-	Y	1	[redacted] 2002; Physical Chemical tests on pure BIT. [redacted] Project 1274163	
3.11 Flammability, including auto-flammability and identity of combustion products (IIA, III 3.8)								
Flammability 1	EC A.10	Purity = 98.0%	BIT is not highly flammable.	It was not possible to ignite the test item in the preliminary test. The main test was therefore not performed.	Y	1	[redacted] 2006; Flammability of [redacted] BIT. Report No. B 053/2006.	
Flammability 2	EC A.16	Purity = 98.0%	No self ignition was observed up to the stop temperature of 400 °C.	-	Y	1	[redacted] 2007; Determination of the Relative Self-Ignition Temperature of [redacted] BIT. Report No. B 004/2007	

Section A3 Physical and Chemical Properties

Annex point IIA, III 3 **PHYSICAL AND CHEMICAL PROPERTIES OF ACTIVE SUBSTANCE**

Subsection (Annex point)	Method	Purity / Specification	Results	Remarks / Justification	GLP (Y/N)	Reliability	Reference	Official use only
Flammability 3	EC A.13	-	-	Refer to TNG Justification for Non-submission of Data A3.11_3.	-	-	-	
3.12 Flash-point (IIA, III 3.9)				Refer to TNG Justification for Non-submission of Data A3.12			None	**X9**
3.13 Surface tension (IIA, III 3.10)	EC A.5	Purity = 98.0%	The surface tension of an aqueous solution (1 g/L) of BIT at 20 °C was 72.6 mN/m. BIT is not a surfactant.	The test was performed using a tensionmeter employing a procedure based on the ring method. The surface tension was calculated from an average of 10 values (2 × 5 values) acquired over 2 tests.	Y	1	[redacted] 2007; Determination of the Surface Tension of an Aqueous Solution of [redacted] BIT. [redacted]. Report No. B 013/2007.	
3.14 Viscosity (-)				Refer to TNG Justification for Non-submission of Data A3.14			None	**X10**
3.15 Explosive properties (IIA, III 3.11)	EC A.14	Purity = 98.0%	Thermal Sensitivity: No Reaction Mechanical Sensitivity (with respect to shock): No Reaction	Thermal sensitivity, mechanical sensitivity (shock) and mechanical sensitivity (friction) tests were performed	Y	1	[redacted] 2007; Determination of the Explosive Properties of [redacted] BIT. Report No. B	

Section A3 Physical and Chemical Properties

Annex point IIA, III 3 **PHYSICAL AND CHEMICAL PROPERTIES OF ACTIVE SUBSTANCE**

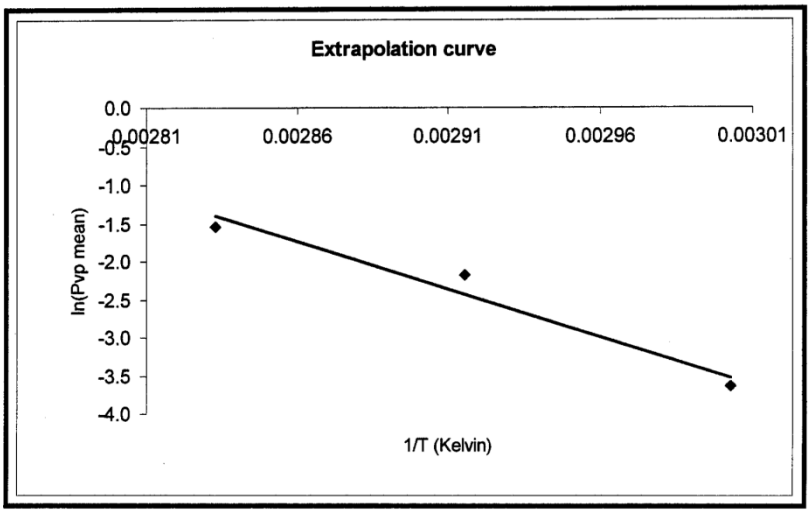
Subsection (Annex point)	Method	Purity / Specification	Results	Remarks / Justification	GLP (Y/N)	Reliability	Reference	Official use only
			Mechanical Sensitivity (with respect to friction): No Reaction BIT has no explosive properties.				003/2007	
3.16 Oxidizing properties (IIA, III 3.12)	EC A.17	Purity = 98.0%	BIT is not classified as an oxidising substance.	Barium nitrate (oxidising substance) was mixed with cellulose at various ratios and the maximum burning rate was determined.	Y	1	[redacted] 2007; Determination of the Oxidizing Properties of [redacted] BIT. Report No. B 001/2007.	**X11**
3.17 Reactivity towards container material (IIA, III 3.13)	EPA 63-20	Technical grade active substance (Purity = > 76%)	No corrosion was observed for aluminium, carbon steel, stainless steel 304, stainless steel 316 or polypropylene. A purplish deposit was observed on the surface of the carbon steel samples and there was a small weight gain (mean value of 0.2% w/w). A slight surface deposit was also observed on one of the aluminium test samples,	Aluminium, carbon steel, stainless steel 304, stainless steel 316 and polypropylene samples were immersed in test substance (two different batches of technical grade active substance were tested) for four weeks in the absence of light at 50°C	Y	1	[redacted].; 1996; Purified [redacted] Physical Chemical Data Package. [redacted] Project 267	

Section A3 Physical and Chemical Properties

Annex point IIA, III 3 **PHYSICAL AND CHEMICAL PROPERTIES OF ACTIVE SUBSTANCE**

Subsection (Annex point)	Method	Purity / Specification	Results	Remarks / Justification	GLP (Y/N)	Reliability	Reference	Official use only
			however no weight change was observed. A weight gain of 0.2% w/w was observed for one of the polypropylene samples.					

Table 3.2-2: Calculation of Vapour Pressure at 20°C



In IVA3.2_2 ([redacted] 1998) tests using the gas saturation method were performed at 60, 70 and 80 °C due to the low vapour pressure of BIT.

Based on the experimental results the vapour pressure curve (In(P_v mean) versus 1/T) was plotted:

Y = b*x+a

Where:

Y = In(P_v mean)

x = 1/T(Kelvin)

b = -12431 (slope)

a = 33.813 (y-axis intercept)

r^2 = 0.9585

Vapour Pressure at 20°C (Calculation performed by the notifier):

In (P_v mean) = (-12431 * 1/293) + 33.813

In (P_v mean) = -8.614

Vapour Pressure = 1.8 x 10^{-4} Pa at 20 °C

Table 3.2.1-1 : Calculation of Henry's Law Constant

Based on the calculation in the TGD the Henry's Law Constant is calculated as follows:-

$$HENRY = \frac{VP * MOLW}{SOL}$$

Vapour pressure = 6.3×10^{-5} Pa at 20°C

Solubility = 1288 mg/L at 20°C

$$HENRY = \frac{6.3 \times 10^{-5} * 151.19}{1288}$$

HENRY = 7.40×10^{-6} Pa.m^3/mol at 20°C

Table A3.4.1-1: Molar Absorption Coefficients for BIT (Purity of > 99%)

Solvent	log ε		
	1st Maximum	2nd Maximum	3rd Maximum
NaOH	4.07 at 222 nm	4.07 at 245nm	3.64 at 308 nm
HCl	4.31 at 225 nm	3.73 at 318 nm	-
H_2O	4.29 at 224 nm	3.72 at 319 nm	-
Methanol	4.23 at 225 nm	3.64 at 316 nm	-

Table A3.4.2-1: Characteristic Resonance of the Infra-Red Spectrum from BIT (Purity of > 99%)

Wave Number (cm^{-1})	Group Bands	Assignment
607	Amide (sec.)	C-C Stretching
743	Amide (sec.)	N-H Deformation
1318-1 325	Amide (sec.)	N-H Deformation
1639	Amide (sec.)	C-O Stretching

Table A3.4.3-1: Data from ^{1}H-NMR-Spectroscopy of BIT (Purity of 99.8%)

Atom	^{1}H Chemical Shift (δ)	^{13}C Chemical Shift (ppm)	Notes for ^{1}H Spectrum
1	8.02	121.4	Doublet of doublets, 1H
2	7.65	130.0	Doublet of triplets, 1H
3	7.47	124.8	Doublet of triplets, 1H
4	7.98	124.1	Doublet of doublets, 1H
5	-	147.4	-
6	-	124.7	-
7	-	164.8	-
8	11.6	-	Singlet, 1H

Table A3.4.3-2: Data from 1H-NMR-Spectroscopy of BIT (Purity of > 99%)

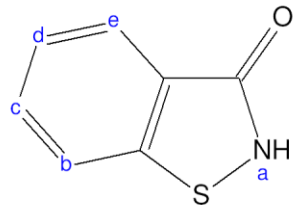
Atom-ID	^{1}H-Chemical Shift (ppm)
	
a	11.59
b,e	8.01 - 8.03
b,e	7.92 - 7.95
c,d	7.63 - 7.68
c,d	7.43 - 7.49
Solvent (DMSO)	2.54 - 2.55

Figure A3.4.2-1: IR- Spectrum from BIT (Purity of > 99%)

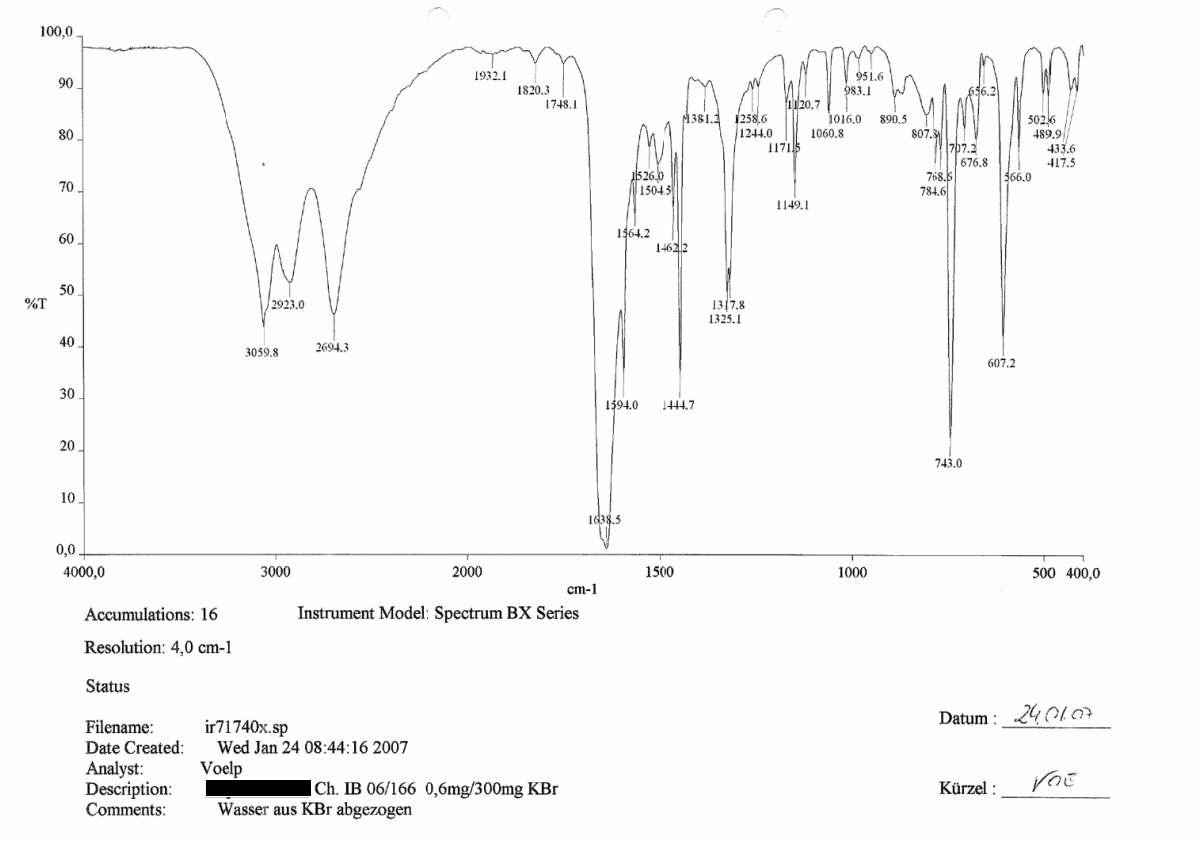


Figure A3.4.3-1: ^{1}H-NMR Spectrum from BIT (Purity of 99.8%)

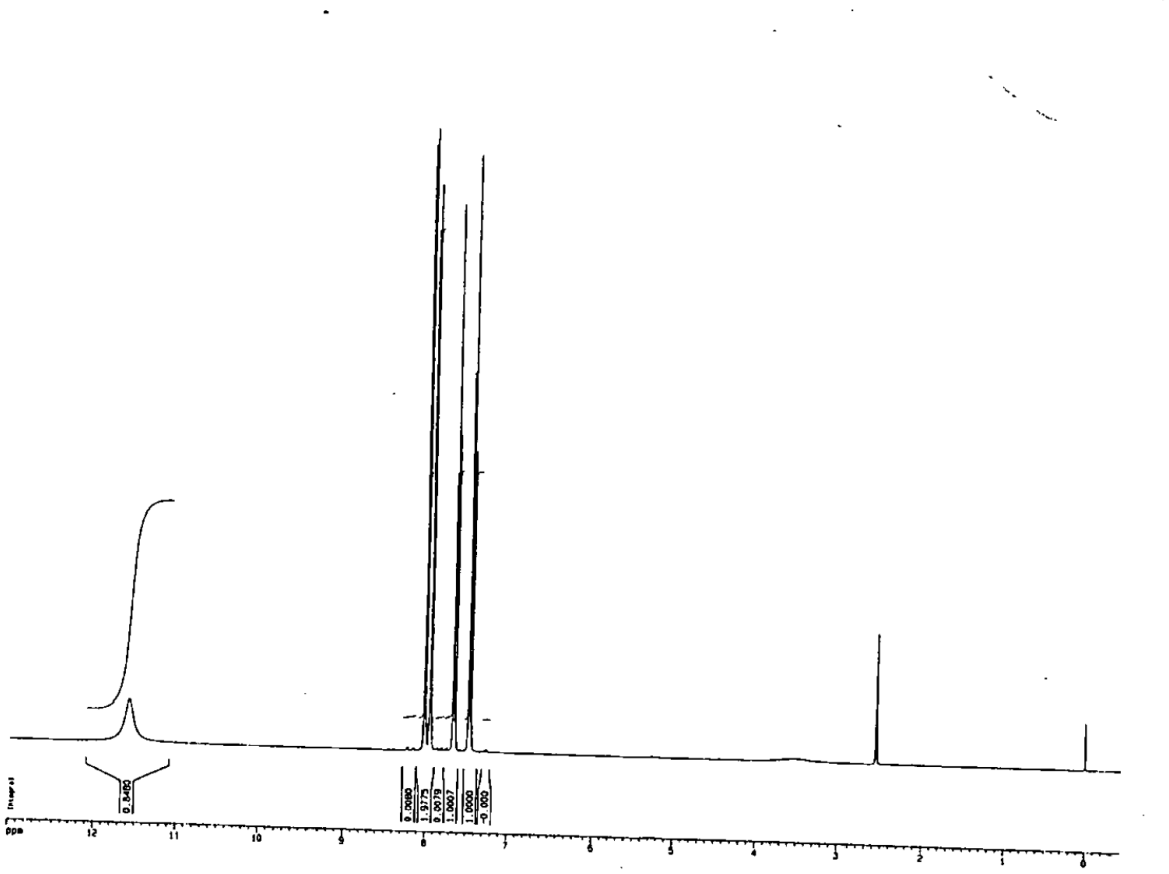


Figure A3.4.3-2: ^{1}H-NMR Spectrum from BIT (Purity of > 99%)

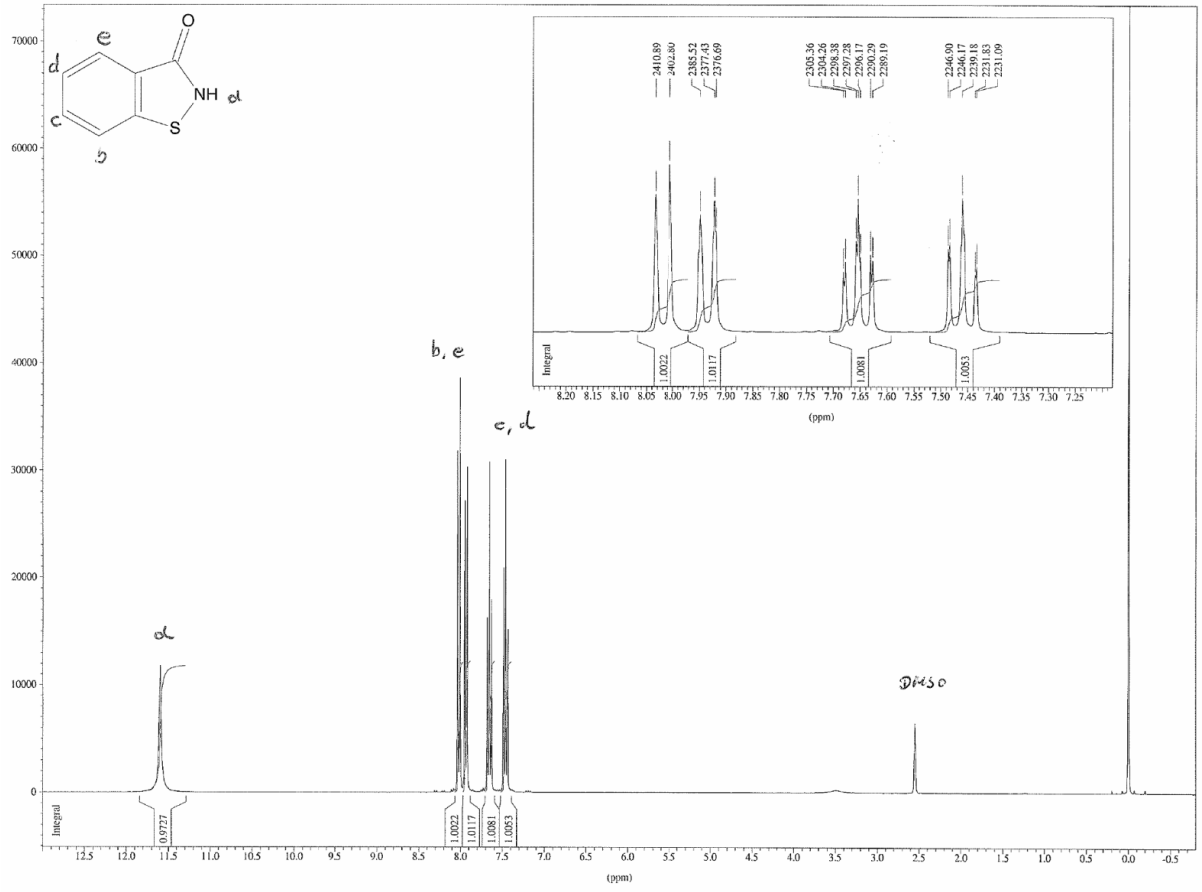
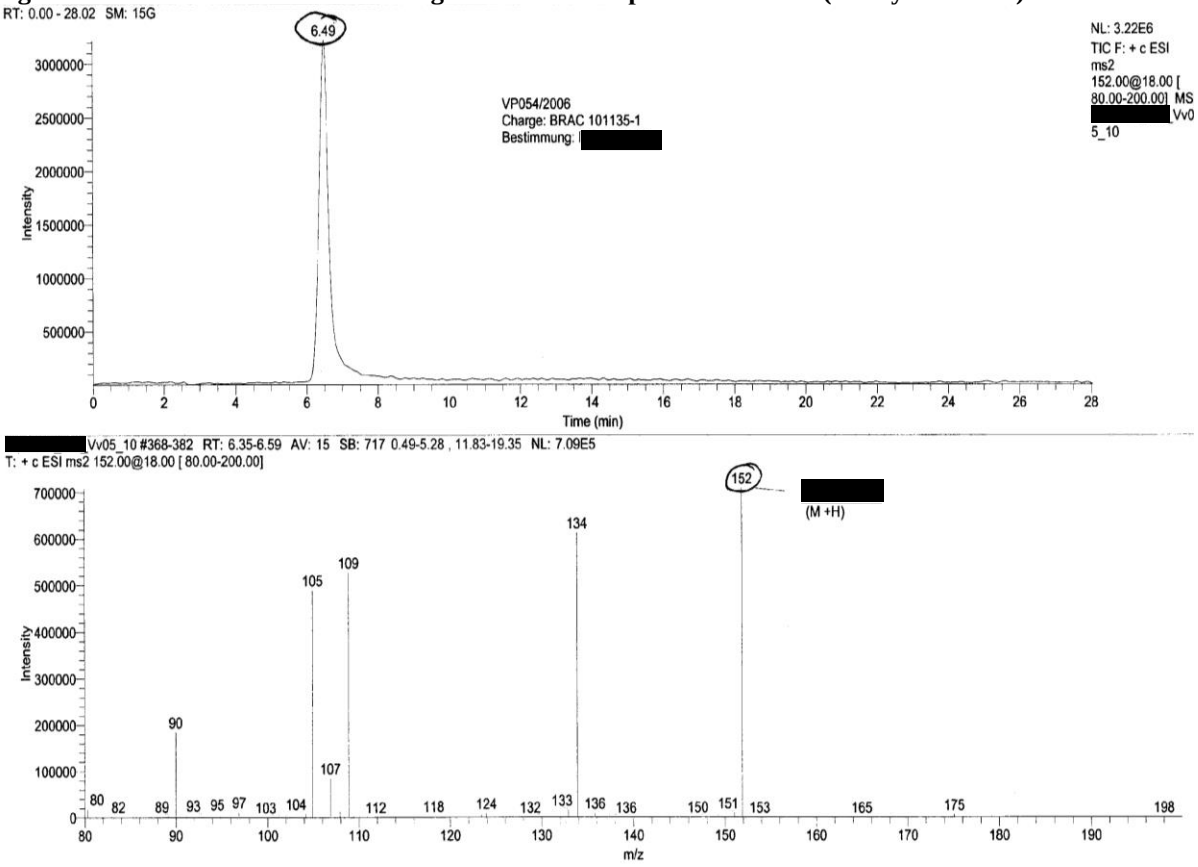


Figure A3.4.4-1: LC-MS Chromatogram and Mass Spectra from BIT (Purity of > 98%)



BIT Retention Time = 6.49

Molecular Ion at 152 m/z ($M^{+\cdot}$+H)

Section A3	**Physical and Chemical Properties**	
Subsection A3.7 **Annex Point IIIA3.1**	**SOLUBILITY IN ORGANIC SOLVENTS, INCLUDING THE EFFECT OF TEMPERATURE ON SOLUBILITY**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ] **Limited exposure [ ]**	**Technically not feasible [ ] Scientifically unjustified [ ]** **Other justification [X]**	
Detailed justification:	An increase in temperature will increase the solubility of BIT. Similarly a decrease in temperature will decrease the solubility, as proved by LeChatelier's Principle whereby all systems are in equilibrium and therefore if temperature is increased or decreased the equilibrium must shift to counteract the change. Solute+Solvent+Heat ↔ Solution From the results shown below for the calculated $LogK_{ow}$, based on water solubility data calculated using EUSES, it can be seen that temperature does not significantly change the solubility of the test substance in octanol or water. Using the relationship between water solubility and octanol/water partition coefficient it is possible to calculate the $LogK_{ow}$ at different temperatures. Equation contained within EPIWINv 3.12:- $LogS\ (mol/L) = (0.796\text{-}0.854)\cdot(LogK_{ow}\text{-}0.00728)(151.19)$	
Undertaking of intended data submission [ ]		

Temperature (ºC)	**Water solubility (mg/L)**	**Moles/L**	**Log Water solubility (LogS)**	**LogKow**
25	1380	9.13E-03	-2.040	2.032
10	1118	7.40E-03	-2.131	2.139
30	1480	9.79E-03	-2.009	1.996

As the test substance is considered to be "of high solubility" in organic solvents a change in temperature from *ca* 10 to 30ºC will not result in a significant change in solubility and the compound will still be classed as "of high solubility".

Section A3	**Physical and Chemical Properties**
Subsection A3.7 **Annex Point IIIA3.1**	**SOLUBILITY IN ORGANIC SOLVENTS, INCLUDING THE EFFECT OF TEMPERATURE ON SOLUBILITY**
	Evaluation by Competent Authorities
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*December 2007*
Evaluation of applicant's justification	*The non-submission of data is justified*
Conclusion	*Acceptable*
Remarks	*No further remarks*

Section A3	**Physical and Chemical Properties**	
Subsection A3.8 **Annex Point IIIA, III.2**	**STABILITY IN ORGANIC SOLVENTS USED IN B.P. AND IDENTITY OF RELEVANT BREAKDOWN PRODUCTS (IIIA3.2)**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ] **Limited exposure [ ]**	**Technically not feasible [ ]** **Scientifically unjustified [ ]** **Other justification [X]**	
Detailed justification:	This is an additional data requirement. The TNsG for Additional Data requirements indicates this endpoint must be provided "if the active substance as manufactured includes an organic solvent". Technical Grade BIT, the active substance as manufactured, does not include an organic solvent, therefore non-inclusion of this additional data requirement is justified on the basis that the criteria for requirement are not met.	
Undertaking of intended data submission [ ]		
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*December 2007*	
Evaluation of applicant's justification	*The non-submission of data is justified*	
Conclusion	*Acceptable*	
Remarks	*No further remarks*	

Section A3	**Physical and Chemical Properties**	
Subsection A3.11_3 **Annex Point IIA III.3.8**	**FLAMMABILITY INCLUDING AUTO-FLAMMABILITY AND IDENTITY OF COMBUSTION PRODUCTS**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ] **Limited exposure [ ]**	**Technically not feasible [ ]** **Scientifically unjustified [X]** **Other justification [ ]**	
Detailed justification:	Test EC A.13 (pyrophoric properties) has been omitted since experience in use shows that negative results would be obtained for BIT. Further, BIT has been tested according to Tests EC A.10 (flammability) and EC A.16 (auto-flammability) and was shown to be not flammable with an autoignition temperature of > 400°C (Refer to Doc. III-A TNG Section 3.11).	
Undertaking of intended data submission [ ]		

Evaluation by Competent Authorities

EVALUATION BY RAPPORTEUR MEMBER STATE

Date	*December 2007*
Evaluation of applicant's justification	*The non-submission of data is justified*
Conclusion	*Acceptable*
Remarks	*No further remarks*

Section A3	**Physical and Chemical Properties**	
Subsection A3.12	**FLASH-POINT**	
Annex Point IIA, III.3.9		
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ]	**Technically not feasible [ ]** **Scientifically unjustified [ ]**	
Limited exposure [ ]	**Other justification [X]**	
Detailed justification:	The TNsG for data requirements indicates this endpoint must be provided "for liquids whose vapours can be ignited". Technical Grade BIT, the active substance as manufactured, is a water-wet paste with no ignitable vapours, therefore non-inclusion of this data requirement is justified on the basis that the criteria for requirement are not met.	
Undertaking of intended data submission [ ]		
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*December 2007*	
Evaluation of applicant's justification	*The non-submission of data is justified*	
Conclusion	*Acceptable*	
Remarks	*No further remarks*	

Section A3	**Physical and Chemical Properties**	
Subsection A3.14	**VISCOSITY**	
Annex Point 3.14		
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ]	**Technically not feasible [ ]** **Scientifically unjustified [x]**	
Limited exposure [ ]	**Other justification [ ]**	
Detailed justification:	This is an additional data requirement. The TNsG for Additional Data requirements indicates "This data is always required for liquid substances, excluding Product-type 5". Technical Grade BIT, the active substance as manufactured, is a water-wet paste, therefore non-inclusion of this additional data requirement is justified on the basis that the criteria for requirement are not met.	
Undertaking of intended data submission [ ]	--	
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*December 2007*	
Evaluation of applicant's justification	*The non-submission of data is justified*	
Conclusion	*Acceptable*	
Remarks	*No further remarks*	

Evaluation by Competent Authorities

EVALUATION BY RAPPORTEUR MEMBER STATE

Date *December 2007*

Applicant´s Comment

Evaluation of data submitted under section A3

3.1.1. Melting point

Materials and Method: The applicant's version is adopted.

Results: The applicant's version is adopted.

Reliability: 1

Acceptability: The method and result are acceptable

3.1.2. Boiling point

Materials and Method: The applicant's version is adopted.

Results: The applicant's version is adopted.

Reliability: 1

Acceptability: The method and result are acceptable

3.1.3. Relative density (1 & 2)

Materials and Method: (X1 y X2) The pycnometer method was used to determine the relative density of the test substance by determining the amount of water displaced by BIT. The applicant's version is adopted.

Results: The applicant's version is adopted.

Reliability: 2

Acceptability: The method and result are acceptable.

3.2. Vapour pressure

Vapour pressure 1

Materials and Method: (X3) The method used was the Knudsen effusion procedure which is a variation of the vapour pressure balance method. The applicant's version is adopted.

Results: The applicant's version is adopted.

Reliability: 1.

Acceptability: The method and result are acceptable.

Vapour pressure 2

Materials and Method: The applicant's version is adopted.

Results: (X4) The vapour pressure was extrapolated by linear regression at 20ºC and 25ºC. The applicant's version is adopted.

Reliability: 2

Acceptability: The method and result are acceptable.

3.2.1. Henry's Law Constant

Materials and Method: The applicant's version is adopted.

Results: The applicant's version is adopted.

Reliability: 1

Acceptability: The method and result are acceptable

3.3. Appearance

Materials and Method: The applicant's version is adopted.

Results: The applicant's version is adopted.

Reliability: 1

Acceptability: The method and result are acceptable

3.4. Absorption spectra, and mass spectrum

3.4.1. UV/VIS

Materials and Method: The applicant's version is adopted.

Results: The applicant's version is adopted.

Reliability: 1

Acceptability: The method and result are acceptable

3.4.2. IR

Materials and Method: The applicant's version is adopted.

Results: The applicant's version is adopted.

Reliability: 2

Acceptability: The method and result are acceptable

3.4.3. NMR

Materials and Method: The applicant's version is adopted.

Results: The applicant's version is adopted.

Reliability: 1

Acceptability: The method and result are acceptable

3.4.4. MS

Materials and Method: The applicant's version is adopted.

Results: The applicant's version is adopted.

Reliability: 1

Acceptability: The method and result are acceptable

3.5. Water solubility

Water solubility 1

Materials and Method: (X5) The solubility was determined with the flask method. The applicant's version is adopted.

Results: The applicant's version is adopted.

Reliability: 1.

Acceptability: The method and result are acceptable.

Water solubility 2

Materials and Method: (X6) The solubility was determined with the flask method. The applicant's version is adopted.

Results: The applicant's version is adopted.

Reliability: 2.

Acceptability: The method and result are acceptable.

Water solubility 3

Materials and Method: The applicant's version is adopted.

Results: The applicant's version is adopted.

Reliability: 1.

Acceptability: The method and result are acceptable.

3.6. Dissociation constant

Materials and Method: The applicant's version is adopted.

Results: The applicant's version is adopted.

Reliability: 1.

Acceptability: The method and result are acceptable.

3.7. Solubility in organic solvents

Materials and Method: The applicant's version is adopted.

Results: The applicant's version is adopted.

Reliability: 2.

Acceptability: The method and result are acceptable.

3.8. Stability in organic solvents used in b.p.

(X7) The non submission of data is justified by the applicant indicating that as the active substance does not include an organic solvent.

3.9 Partition coefficient

Log P_{ow} 1

Materials and Method: The applicant's version is adopted.

Results: The applicant's version is adopted.

Reliability: 1.

Acceptability: The method and result are acceptable

Log P_{ow} 2

Materials and Method: The applicant's version is adopted..

Results: (X8) The results are acceptable although the results are obtained by calculations.

Reliability: 2.

Acceptability: The method and result are acceptable.

3.10 Thermal stability

Materials and Method: The applicant's version is adopted.

Results: The applicant's version is adopted.

Reliability: 1

Acceptability: The method and result are acceptable

3.11 Flammability

Flammability 1

Materials and Method: The applicant's version is adopted.

Results: The applicant's version is adopted.

Reliability: 1

Acceptability: The method and result are acceptable

Flammability 2

Materials and Method: The applicant's version is adopted.

Results: The applicant's version is adopted.

Reliability: 1

Acceptability: The method and result are acceptable

3.12. Flash point

(X9) The non submission of data is justified because the active substance as manufactured is a water-wet paste with no ignitable vapours.

3.13. Surface tension

Materials and Method : The applicant's version is adopted.

Results: The applicant's version is adopted.

Reliability: 1

Acceptability: The method and result are acceptable

3.14. Viscosity

(X10) The non submission of data is justified because the active substance as manufactured is a water-wet paste with no ignitable vapours.

3.15. Explosive properties

Materials and Method : The applicant's version is adopted.

Results: The applicant's version is adopted.

Reliability: 1

Acceptability: The method and result are acceptable

3.16. Oxidizing properties

Materials and Method : The applicant's version is adopted.

Results: The applicant's version is adopted.

Reliability: 1

Acceptability: The method and result are acceptable. (X11) The single results from all tests are summarized in the following table:

mixture	***Maximum burning rate (mm/s)***
Oxidiser / Cellulose	***1.24***
Test item / Cellulose	***0.92***

3.17. Reactivity towards the container

Materials and Method: The applicant's version is adopted.

Results: The applicant's version is adopted.

Reliability: 1

Acceptability: The method and result are acceptable.

Section A4 Analytical Methods for Detection and Identification

Subsection A4.1/1

Annex Point IIA, IV 4.1 (a)

ANALYTICAL METHOD FOR THE DETERMINATION OF 1,2-BENZISOTHIAZOL-3-(2*H*)-ONE IN THE ACTIVE SUBSTANCE AS MANUFACTURED

BIT in [redacted] press Paste

			Official use only
		1 REFERENCE	
1.1	**Reference**	No Author; 2006; Analytical Method for the Determination of 1,2-Benziosthiazolin-3-one in [redacted] Formulations. [redacted] Project No. 1289536.	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Arch Chemicals Inc	
1.2.2	Companies with letter of access	None	
1.2.3	Criteria for data protection	Data on existing substance for first entry into Annex I	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	EPA Guideline Ref: 830.1700	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Preliminary test**	Not applicable.	
3.1.1	Enrichment	**Method Validation for Determination of BIT** <u>Preparation of BIT Quality Control Standard Solutions using BIT Analytical Reference Standard</u> 0.0795 g, 0.1252 g and 0.1012 g of BIT analytical reference material (ODAS 0235.04) were weighed accurately into l00 mL volumetric flasks. 10 mL methanol and 2.5 mL acetic acid were then added and the contents of the flask were sonicated. The solution was allowed to return to room temperature and made up to volume with water. The above solutions were diluted as follows: 5.0 mL into 50 mL* volumetric flask diluted to the mark volume with methanol : acetic acid : distilled deionised water (10 : 2.5 : 87.5). (*Note there appears to be discrepancy in the report. The text suggests that there is a dilution factor of 1000 however according to the data the dilution factor should be 500 (Refer to Section 4.2).	

Section A4 Analytical Methods for Detection and Identification

Subsection A4.1/1

Annex Point IIA, IV 4.1 (a)

ANALYTICAL METHOD FOR THE DETERMINATION OF 1,2-BENZISOTHIAZOL-3-(2*H*)-ONE IN THE ACTIVE SUBSTANCE AS MANUFACTURED

BIT in [redacted] press Paste

3.1.2 Cleanup	Not applicable
3.2 Detection	
3.2.1 Separation Method	**Method Validation for Determination of BIT** Instrument: Alliance 2690 HPLC Column: Spherisorb S5ODS-1 (25 cm x 3.2 mm, 5 µm) Column Temperature: 40 °C Injection Volume: 10 µL Mobile Phase (Isocratic): Distilled Deionised Water : Acetonitrile : Methanol: Acetic Acid (77.5:11:9:2.5) Flow Rate: 0.75 mL/min Run Time: 20 minutes
3.2.2 Detector	**Method Validation for Determination of BIT** Detector: UV at 254 nm, collecting 190 - 400 nm
3.2.3 Standard(s)	**Method Validation for Determination of BIT** Validation Phase: 1. Calibration Standard: BIT was determined by external standard comparison against BIT Analytical Reference standard (BIT reference material ODAS 0235.04) The reference material was weighed accurately (refer to the table below for weights) into a 100 mL volumetric flask. 10 mL methanol and 2.5 mL acetic acid were added and the contents of the flask were sonicated. The solution was allowed to return to room temperature and then diluted to the mark with water. The solution was further diluted as follows: 5.0 mL added to a 50 mL* volumetric flask and made to volume with methanol : acetic acid : distilled deionised water (10 : 2.5 : 87.5). (*Error in the report, refer to Section 4.2). The concentration of the individual calibration standards are detailed in the following table:

Weight (g)	Concentration (mg/mL)[a]	Concentration of Diluted Solution % w/v

Section A4 Analytical Methods for Detection and Identification

Subsection A4.1/1

Annex Point IIA, IV 4.1 (a)

ANALYTICAL METHOD FOR THE DETERMINATION OF 1,2-BENZISOTHIAZOL-3-(2*H*)-ONE IN THE ACTIVE SUBSTANCE AS MANUFACTURED

BIT in [redacted] press Paste

0.0253	0.0506	0.00506
0.0498	0.0996	0.00996
0.1010	0.2020	0.02020
0.1495	0.2990	0.02990
0.1983	0.3966	0.03966

[a] Calculated using a dilution factor of 500 mL

Peak areas were obtained for each calibration standard (analysis was performed using the HPLC conditions detailed in Section 3.2.1) and a calibration curve was obtained by weighted least squares linear regression analysis (1/x) of the plot of the peak area versus the concentration of BIT in each calibration standard.

2. Quality Control Standard:

Analytical Grade BIT (ODAS 0235.04) was used to prepare quality control standard solutions. Refer to Section 3.1.1 for details.

3.2.4 Interfering susbtance(s)

Method Validation for Determination of BIT

There were no substances observed which co-eluted with BIT. Refer to Section 3.4.

3.3 Linearity

3.3.1 Calibration range

Method Validation for Determination of BIT

Validation Phase

Calibration Range: 0.0506 mg/mL to 0.3966 mg/mL (0.00506 % w/v to 0.03966 % w/v)

Section A4 Analytical Methods for Detection and Identification

Subsection A4.1/1

Annex Point IIA, IV 4.1 (a)

ANALYTICAL METHOD FOR THE DETERMINATION OF 1,2-BENZISOTHIAZOL-3-(2*H*)-ONE IN THE ACTIVE SUBSTANCE AS MANUFACTURED

BIT in [redacted] press Paste

3.3.2 Number of measurements	**Method Validation for Determination of BIT** Validation Phase One injection at 5 different concentrations.
3.3.3 Linearity	**Method Validation for Determination of BIT** Correlation coefficient (r^2) = 1.000
3.4 Specificity: interfering substances	**Method Validation for Determination of BIT** Validation Phase Blank samples of the diluent used to prepare the analytical reference standards were analysed and no co-eluting interferences were reported. The specificity of the analytical method for determination of BIT in [redacted] formulation was demonstrated by comparison of the retention time and spectrum of a [redacted] formulation sample to BIT analytical reference material. There were no significant differences observed.
3.5 Recovery rates at different levels	**Method Validation for Determination of BIT** Validation Phase Quality control standards were prepared at the following concentrations:

Level	QC Std ID	Concentration (mg/mL)	Concentration of Diluted Solution (% w/v)
1	1	0.1590	0.01590
2	3	0.2024	0.02024
3	2	0.2504	0.02504

Section A4 Analytical Methods for Detection and Identification

Subsection A4.1/1

Annex Point IIA, IV 4.1 (a)

ANALYTICAL METHOD FOR THE DETERMINATION OF 1,2-BENZISOTHIAZOL-3-(2*H*)-ONE IN THE ACTIVE SUBSTANCE AS MANUFACTURED

BIT in [redacted] press Paste

The Quality Control standards were injected in triplicate and the concentration was determined using a five point calibration line.

The percentage recovery of each injection was determined. The accuracy is detailed in the following table:

QC Std ID	Conc.[1] (% w/v)	Calculated Conc. [1] (% w/v)	Recovery (%)	Mean Recovery (%)	Overall Recovery
1	0.01590	0.01589	99.96	99.96	100.31
	0.01590	0.01585	99.71		
	0.01590	0.01591	100.05		
3	0.02024	0.02037	100.63	100.63	
	0.02024	0.02046	101.06		
	0.02024	0.02037	100.65		
2	0.02504	0.02510	100.24	100.24	
	0.02504	0.02514	100.41		
	0.02504	0.02506	100.07		

[1]Concentration of BIT analytical reference standard.

3.5.1 Relative standard deviation

Method Validation for Determination of BIT

Validation Phase

The relative standard deviation (coefficient of variation, %) at each concentration and the overall relative standard deviation for all injections are presented in the following table:

QC Std ID	Conc.[1] (% w/v)	RSD (%)	Overall RSD (%)
Std 1	0.01590	0.18	0.42

Section A4 Analytical Methods for Detection and Identification

Subsection A4.1/1

Annex Point IIA, IV 4.1 (a)

ANALYTICAL METHOD FOR THE DETERMINATION OF 1,2-BENZISOTHIAZOL-3-(2*H*)-ONE IN THE ACTIVE SUBSTANCE AS MANUFACTURED

BIT in [redacted] press Paste

Std 1	0.01590		
Std 1	0.01590		
Std 3	0.02024		
Std 3	0.02024	0.24	
Std 3	0.02024		
Std 2	0.02504		
Std 2	0.02504	0.17	
Std 2	0.02504		

[1]Concentration of BIT analytical reference standard

3.6 Limit of determination

Method Validation for Determination of BIT

The limit of determination of the validated method is 0.00506 % w/v BIT (0.0506 mg/mL).

3.7 Precision --

3.7.1 Repeatability

Method Validation for Determination of BIT

Validation Phase

1. Precision Data for Quantification of BIT in BIT Analytical Reference Standard :

Repeatability for quantification of BIT was demonstrated. The RSD for 3 replicates at 0.01590, 0.02024 and 0.02504% w/v (0.159, 0.2024 and 0.2504 mg/mL) were all < 0.3%.

The overall RSD for 3 replicates at each concentration was < 0.5%.

2. System Precision

System precision was determined by the duplicate injection of 6 standard solutions at the concentration of 0.0202 % BIT w/v (0.202 mg/mL BIT). The detector response (Area) was normalised for each injection and the RSD was calculated.

RSD for system precision = 0.38%

3.7.2 Independent laboratory validation

Not Applicable

Section A4 Analytical Methods for Detection and Identification

Subsection A4.1/1

Annex Point IIA, IV 4.1 (a)

ANALYTICAL METHOD FOR THE DETERMINATION OF 1,2-BENZISOTHIAZOL-3-(2*H*)-ONE IN THE ACTIVE SUBSTANCE AS MANUFACTURED

BIT in [redacted] press Paste

4 APPLICANT'S SUMMARY AND CONCLUSION

4.1 Materials and methods

Method Validation for Determination of BIT

Validation Phase

A method was validated for the determination of BIT by HPLC with UV detection at 254 nm. A Spherisorb S5ODS-1 (5 µm, 25 cm x 3.2 mm, 5 µm) HPLC column maintained at 40 °C was employed with water:acetonitrile:methanol:acetic acid (77.5:11:9:2.5) mobile phase under isocratic conditions and a run time of 20 minutes.

Quality control standards were prepared using BIT analytical standard at three concentrations over the range BIT 0.0159 to 0.02504 % w/v (0.159 to 0.2504 mg/mL) BIT. The quality control standards were injected (10 µL) in triplicate and quantified using BIT analytical reference material as an external standard. Linearity was acceptable over the range of 0.00506% w/v to 0.03966 % w/v with a coefficient of determination (r^2) of 1.000.

The accuracy of the method was determined by comparison of the calculated concentration to the theoretical concentration of the quality control standards. The mean accuracy at 0.01590, 0.0202 to 0.02504 % w/v was 99.96, 100.6 and 100.2%, respectively and the overall accuracy was 100.3%. (The precision of the method was also demonstrated with values of > 0.3% RSD at each concentration and an overall value of 0.42% RSD).

System precision (repeatability) was demonstrated by duplicate injections of six standard solutions at a nominal concentration of 0.02% w/v. The %RSD for this analysis was 0.38 and therefore system precision was acceptable.

Solution stability was determined by analysis of an analytical standard at a nominal concentration of 0.02% w/v after storage at room temperature for 24 and 48 hours. Stability of BIT in methanol:acetic acid:distilled deionised water (10:2.5:87.5) for 48 hours at room temperature was demonstrated when compared to freshly prepared solutions.

System suitability was demonstrated by six replicate injections of a standard solution at a nominal concentration of 0.02 % w/v. The percentage RSD of the retention time and detector response (area) of each injection was calculated and found to be acceptable with a value of < 0.7%.

4.2 Conclusion

Validation of an Analytical Method for the Determination of BIT Concentration in Analytical Grade BIT

Validation Phase

Section A4 Analytical Methods for Detection and Identification

Subsection A4.1/1

Annex Point IIA, IV 4.1 (a)

ANALYTICAL METHOD FOR THE DETERMINATION OF 1,2-BENZISOTHIAZOL-3-(2*H*)-ONE IN THE ACTIVE SUBSTANCE AS MANUFACTURED

BIT in [redacted] press Paste

		The analytical method for the determination of the concentration of BIT in analytical grade BIT reference material was found to be acceptable in terms of accuracy, precision, linearity and system suitability.
4.2.1	Reliability	1
4.2.2	Deficiencies	No. The study meets the criteria of EPA Guideline Ref: 830.1700.

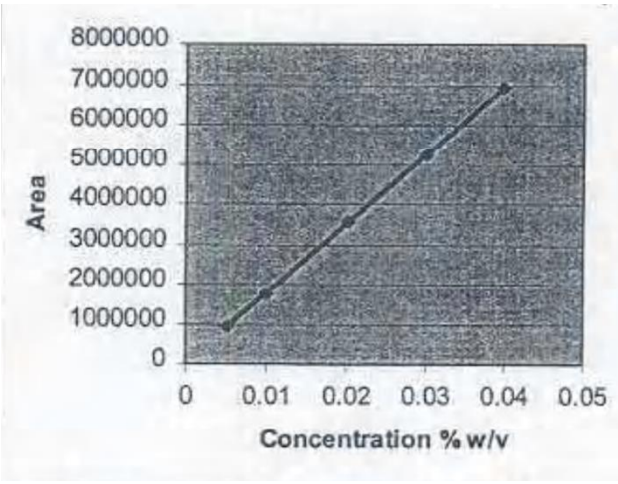
Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*December 2007*
Materials and methods	*The applicant's version is adopted.*
Conclusion	*The applicant's version is adopted.*
Reliability	*1*
Acceptability	*The method and result are acceptable.*
Remarks	*No further remarks*

Table A4_1(1)-1: Method Validation for Determination of BIT: Accuracy and Precision Data for Determination of Concentration of BIT in Analytical Grade Standard

Concentration (% w/v)	Calculated Concentration (% w/v)	Recovery (%)	Mean Recovery (%)	Overall Recovery (%)	Mean RSD (%)	Overall RSD (%)
0.01590	0.01589	99.96	99.96	100.31	0.18	0.42
0.01590	0.01585	99.71				
0.01590	0.01591	100.05				
0.02024	0.02037	100.63	100.63		0.24	
0.02024	0.02046	101.06				

0.02024	0.02037	100.65				
0.02504	0.02510	100.24	100.24		0.17	
0.02504	0.02514	100.41				
0.02504	0.02506	100.07				

Figure A4_1(1)-1: Method Validation for Determination of BIT: Linearity Data



Y = 173281521.4x + 48047.638

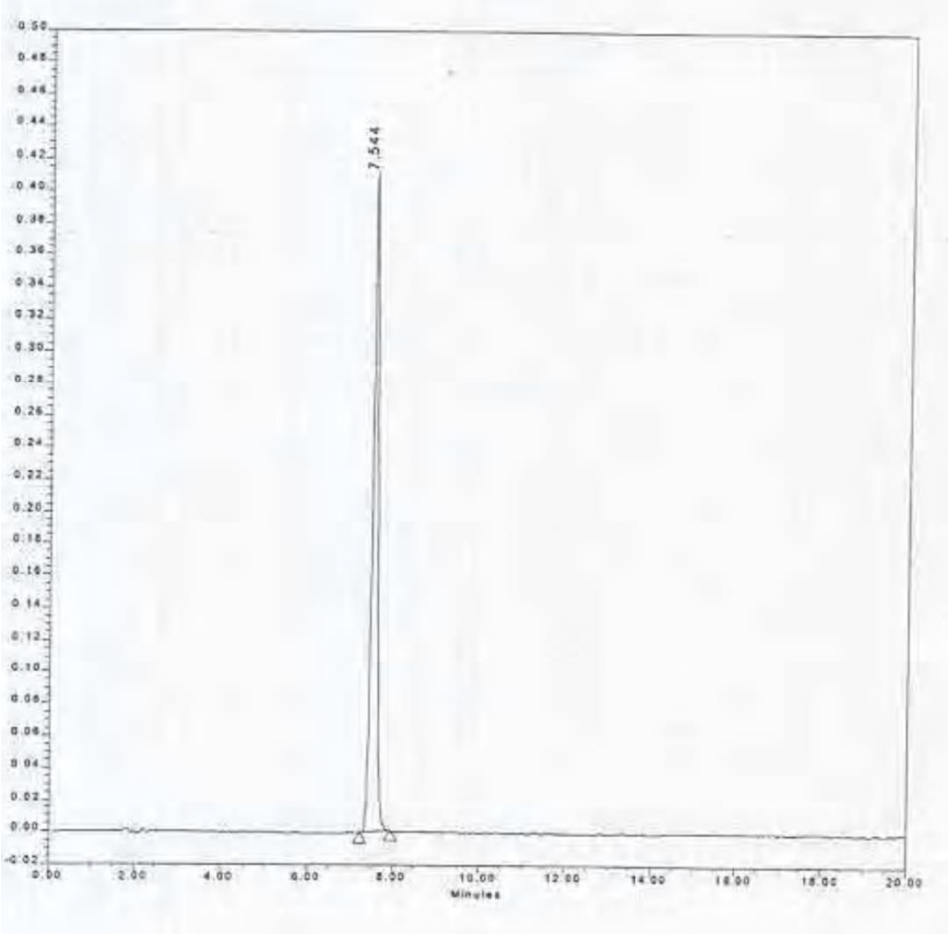
Slope: 173281521.4

Intercept: 48047.638

Concentration (mg/mL)	Concentration (% w/v)	Area	Calculated Concentration (% w/v)[1]
0.0506	0.00506	936289	0.00513
0.0996	0.00996	1764515	0.00991
0.2020	0.02020	3554639	0.02024
0.2990	0.02990	5204705	0.02976
0.3966	0.03966	6936528	0.03975

[1] Calculated using the formula: x = (y-c) /m, Concentration = (Response – Intercept)/Slope

Figure A4_1(1)-2: Method Validation for Determination of BIT: Typical HPLC Chromatogram of [redacted] Formulation



Section A4.1(4) Analytical Methods for Detection and Identification

Annex Point IIA, IV.4.1 BIT

			Official use only
		1 REFERENCE	
1.1	**Reference**	[redacted]. (2015) 5-Batch Analysis of 1,2-Benzisothiazol-3(2H)-one, [redacted] 511177	
1.2	**Data protection**	Yes	
Data owner		Laboratorios Miret, S.A	
Companies with a letter of Access		Not applicable	
Criteria for data protection		Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I/IA.	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	European Commission: Guidance for Generating and Reporting Methods of Analysis in Support of Pre- and Post-registration Data Requirements for Annex II (part A, Section 4) and Annex III (part A, Section 5) of Directive 91/414, SANCO/3030/99 rev. 4 (11/07/00). Guidance on regulation (EU) No 528/2012 concerning the making available on the market and use of biocidal products (BPR), Version 1.1 November 2014	
2.2	**GLP**	Yes	
2.3	**Deviations**	Not applicable.	
		3 MATERIALS AND METHODS	
3.1	**Preliminary treatment**	This summary details a BIT Analytical Method Validation study.	
3.1.1	Enrichment	**Validation** Analysis of BIT by HPLC with UV Detection **Preparation of solutions** All solutions containing the analytical standard were corrected for purity. Stock solutions 1,2-Benzisothiazol-3(2H)-one: Two stock solutions of the analytical standard 1,2-Benzisothiazol-3(2H)-one were prepared in volumetric flask of 50 ml at concentrations of 995 and 997 mg/l. 10 ml methanol was added to the volumetric flasks. In order to dissolve the test item the solutions were ultrasonicated for 5 minutes. Thereafter the volumetric flask was filled to the mark with 55/45 v/v methanol/water.	

Section A4.1(4) Analytical Methods for Detection and Identification

Annex Point IIA, IV.4.1 BIT

3.1.2 Cleanup

3.2 Detection

Separation method

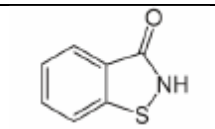
Validation
Analysis of BIT by HPLC with UV Detection
Instrument: Alliance Separation Module 2695 (Waters, Milford, MA, USA)
Column: Symmetry Shield C18, 250mm x 4.6 mm i.d., dp = 5 µm (Waters)
Column temperature: 21°C ± 1°C
Mobile phase: 55/45 (v/v) methanol/water
Flow rate: 1 ml/min
Injection volume: 10 µl
UV, wavelength 254 nm

Detector

Validation
Analysis of BIT by HPLC with UV Detection
Dual λ Absorbance Detector 2487 (Waters)

Standard(s)

Validation
Analysis of BIT by HPLC with UV Detection
Analytical Standard

Identification number	[redacted]
Container	A1
Identification	1,2-Benzisothiazol-3(2H)-one
CAS Number	2634-33-5
Molecular structure	
Molecular formula	C7H5NOS
Molecular weight	151.18
Appearance	Faint yellow powder
Batch	[redacted]
Purity	99.9 %
Storage conditions	At room temperature
Stable under storage conditions until	06 March 2016
Supplier	LAMIRSA, Barcelona, Spain
Article number	561487 (Sigma Aldrich)

Calibration solutions
From two 1,2-Benzisothiazol-3(2H)-one stock solutions, five calibration solutions in the concentration range 6 – 20 mg /l were prepared. The end solution of the calibration solutions was 55/45 v/v methanol/water.

Interfering substance(s)

3.3 Linearity

Section A4.1(4) Analytical Methods for Detection and Identification

Annex Point IIA, IV.4.1

BIT

Calibration range

Validation
Analysis of BIT by HPLC with UV Detection
The calibration line was constructed using all data points. Each of the individual calibration points deviated less than 15% from the calibration line. There was a linear relationship between response and analytical standard concentration in the range of 5.99 – 20.0 mg/l 1,2-Benzisothiazol-3(2H)-one (in end solution). Since the correlation coefficient (r) was > 0.99, the calibration line was accepted.

Number of measurements

Validation
Analysis of BIT by HPLC with UV Detection
Calibration solutions were analysed in duplicate.

Linearity

Validation
Analysis of BIT by HPLC with UV Detection

Slope	2.18×10^4
Intercept	9.69×10^2
Weighting factor	$1/\text{concentration}^2$
r	0.9997

3.4 Specifity: interfering substances

Validation
Analysis of BIT by HPLC with UV Detection
The chromatogram of the calibration solution showed one major peak. The area of this peak was used as response for in the calculations of 1,2-Benzisothiazol-3(2H)-one.
The chromatogram of the blank sample showed no peak at the retention time of 1,2-Benzisothiazol-3(2H)-one. Since no interferences were detected, the specificity requirements were met and the analytical method was found to be specific for the test item.
Identification of 1,2-Benzisothiazol-3(2H)-one was confirmed based on retention time and UV spectrum.

3.5 Recovery rates at different levels

Validation
Analysis of BIT by HPLC with UV Detection
See confidential Doc IIIA Section 4.1(4).

Relative standard deviation

Validation
Analysis of BIT by HPLC with UV Detection
The relative standard deviation (RSD) of the test samples and the predicted % RSD_R and % RSD_r are given below. Since the % RSD for the analysis of 1,2-Benzisothiazol-3(2H)-one was less than the predicted % RSD_r the results of the test samples were considered precise.

% RSD_R	2.0
% RSD_r	1.4
% RSD of the test samples	1.2

3.6 Limit of determination

Validation
Analysis of BIT by HPLC with UV Detection
The limit of determination can be defined as the lowest calibration point covered by the linear calibration curve, for BIT 6mg/l.

3.7 Precision

Section A4.1(4) Analytical Methods for Detection and Identification

Annex Point IIA, IV.4.1 BIT

Repeatability: Validation

Analysis of BIT by HPLC with UV Detection

As detailed for the relative standard deviation validation phase

Independent laboratory validation: Not applicable

4 APPLICANT'S SUMMARY AND CONCLUSION

4.1 Materials and methods

Materials and Methods

Summary of Results

An analytical method based on liquid chromatography using ultraviolet detection was validated for determination of 1,2-Benzisothiazol-3(2H)-one (HPLC-UV).

Validation
Summary of Result
Analysis of BIT by HPLC with UV Detection

	HPLC-UV
	1,2-Benzisothiazol- 3(2H)-one
Specificity	specific
Calibration curve	r = 0.9997
Accuracy	Not required

4.2 Conclusion

The analytical method was found to be suitable only for the quantitative determination of 1,2-Benzisothiazol-3(2H)-one.

Reliability: 1

Deficiencies: None

Evaluation by Competent Authorities

EVALUATION BY RAPPORTEUR MEMBER STATE

Date *October 2020*

Materials and methods *The applicant's version is adopted.*

Conclusion *The applicant's version is adopted.*

Section A4.1(4) Analytical Methods for Detection and Identification

Annex Point IIA, IV.4.1 BIT

Reliability	*1*
Acceptability	*The applicant's version is accepted.*
Remarks	*No further remarks.*

Figure1 Regression line: response of 1,2-Benzisothiazol-3(2H)- one as a functiona of concentration [cal. curve id. 1629].

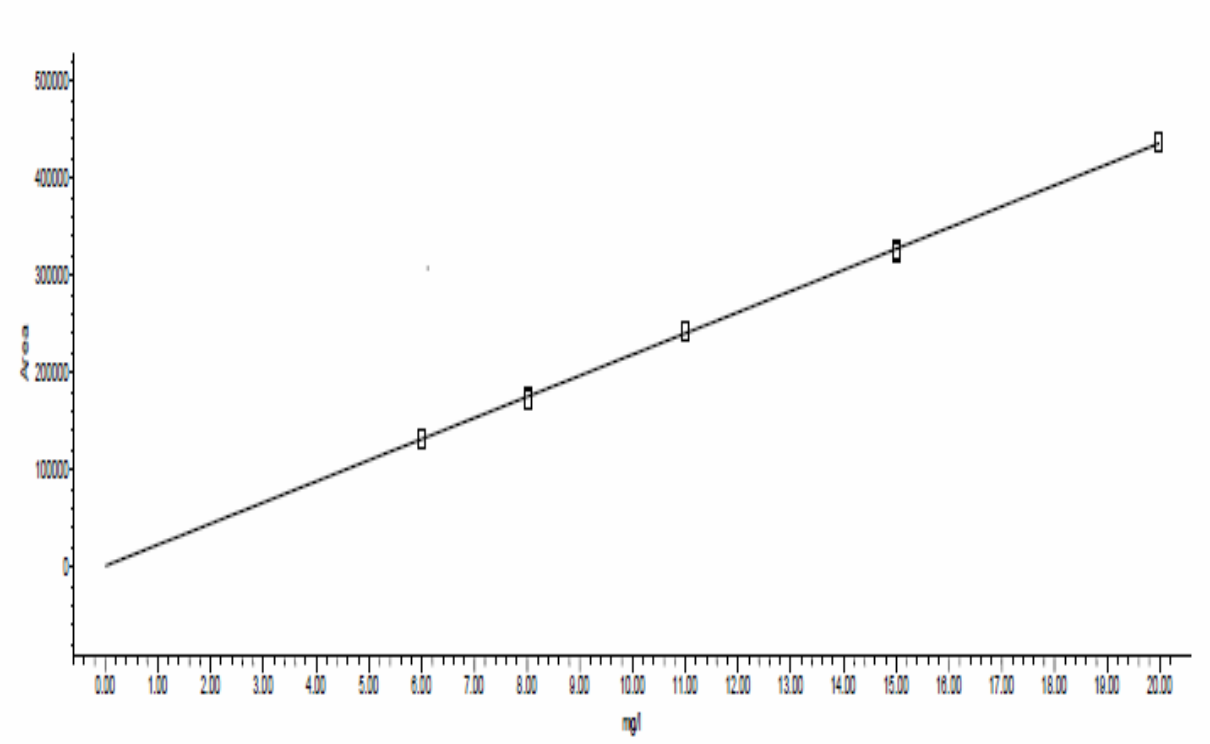


Figure 2 HPLC-UV chromatogram of a blank solution [res. id. 2243].

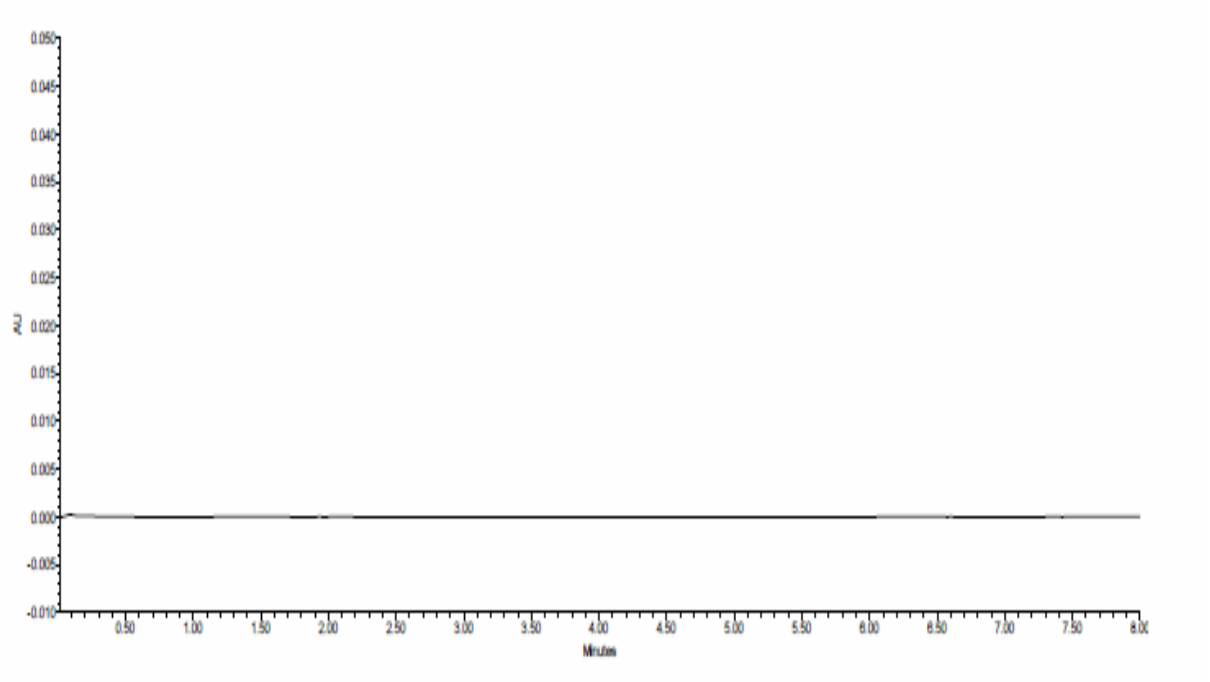
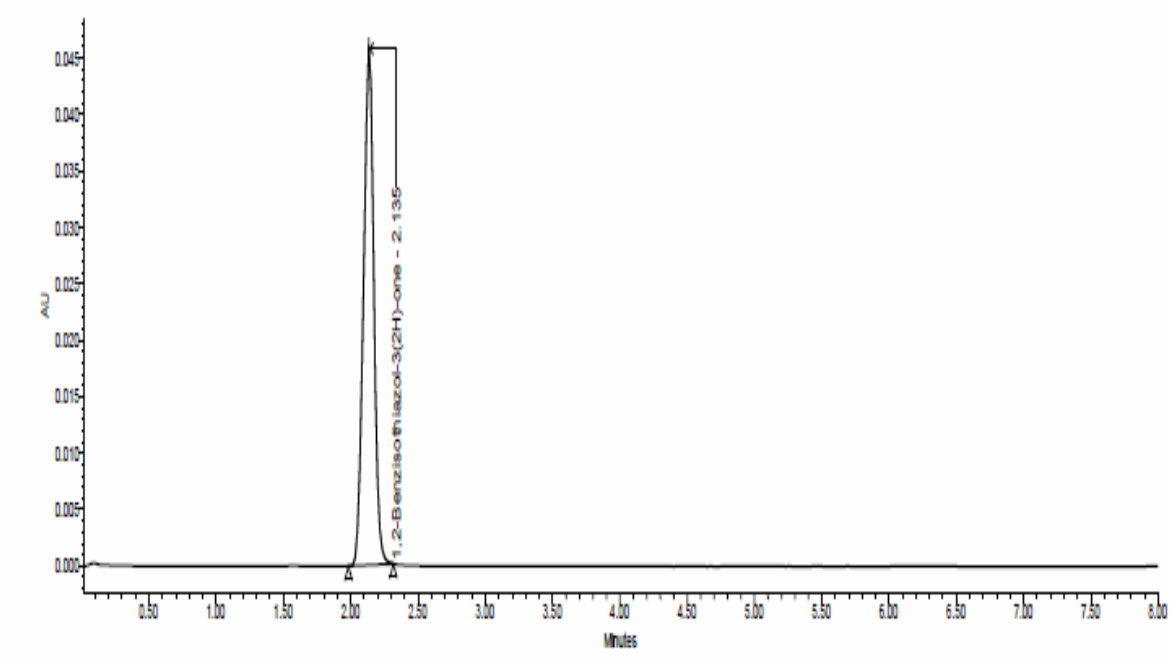


Figure 3 HPLC-UV chromatogram of a 11.0 mg/l calibration solution 1,2-Benzisothiazol-3(2H)- one [res. id. 2238].



Section A4 Analytical Methods for Detection and Identification

Subsection A4.1/3

Annex Point IIA, IV 4.1 (a)

ANALYTICAL METHOD FOR THE DETERMINATION OF 1,2-BENZISOTHIAZOL-3-(2*H*)-ONE IN THE ACTIVE SUBSTANCE AS MANUFACTURED

BIT

			Official use only
		1 REFERENCE	
1.1	**Reference**	[redacted] 1999; Validation of an Analytical Method for [redacted] BIT and Analysis of Five Batches. [redacted] Project 701763; GLP; Unpublished	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Thor GmbH	
1.2.2	Companies with letter of access	None	
1.2.3	Criteria for data protection	Data on existing substance for first entry into Annex I.	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	EPA Guideline Ref: 830.1700 and 830.1800	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Preliminary test**	Confidential data has been removed from this summary. Refer to the Confidential File, Section IIIA 4.1(3) where this summary is presented in full.	
3.1.1	Enrichment	**Validation** Analysis of BIT by HPLC with UV Detection Approximately 180 mg of test item ([redacted] BIT Batch [redacted], approximately 70% active ingredient content) was dissolved in 50 mL acetonitrile:water (80:20, v/v) to prepare a solution of approximately 2.5 mg/mL BIT (active ingredient).	
3.1.2	Cleanup	Not applicable	
3.2	**Detection**		

Section A4 Analytical Methods for Detection and Identification

Subsection A4.1/3

Annex Point IIA, IV 4.1 (a)

ANALYTICAL METHOD FOR THE DETERMINATION OF 1,2-BENZISOTHIAZOL-3-(2*H*)-ONE IN THE ACTIVE SUBSTANCE AS MANUFACTURED

BIT

3.2.1 Separation Method

Validation

Analysis of BIT by HPLC with UV Detection

Apparatus:

Merck-Hitachi Model D-7000 Workstation

Merck Pump L-7100

Merck Autosampler L-7300

Merck Column Oven L-7300

Column: LiChrosphere 100 RP 18e, 250 mm × 4 mm, 5 µm

Mobile Phase

Eluent A: 0.05 % H_3PO_4

Eluent B: Acetonitrile

Gradient:

Time	% A	% B
0-2	90	10
2 -25	10	90
25-35	10	90

Column Temperature: 40 °C

Flow: 1.0 mL/min

Detection: 350 nm

Injection Volume: 10 µL

3.2.2 Detector

Validation

Analysis of BIT by HPLC with UV Detection

Merck DAD Detector L-7450

UV Detection at 350 nm

3.2.3 Standard(s)

Validation

Analysis of BIT

Identity: BIT-Analytical Standard

Batch: [redacted]

Section A4 Analytical Methods for Detection and Identification

Subsection A4.1/3

Annex Point IIA, IV 4.1 (a)

ANALYTICAL METHOD FOR THE DETERMINATION OF 1,2-BENZISOTHIAZOL-3-(2*H*)-ONE IN THE ACTIVE SUBSTANCE AS MANUFACTURED

BIT

	Purity: 100% Expiration Date: August 1999 Approximately 25 mg of [redacted] BIT Standard (100%) was accurately weighed and dissolved in 10 mL of acetonitrile:water (80:20, v/v) to prepare a stock solution of approximately 2.5 mg/mI [redacted] BIT Standard (100%). This stock solution was diluted with acetonitrile:water (80:20, v/v) to prepare calibration standards of approximately 2.3 to 1.7 mg/mL BIT.
3.2.4 Interfering susbtance(s)	Not applicable.
3.3 Linearity	
3.3.1 Calibration range	**Validation** Analysis of BIT Range of approximately 1.7 to 2.5 mg/mL BIT in acetonitrile:water (80:20, v/v). The calibration curve was plotted in area counts versus concentration and linear regression and least square fit was applied to the data.
3.3.2 Number of measurements	**Validation** Analysis of BIT One calibration standard at each of the five concentrations was injected for each calibration line. Three calibration lines were injected over the course of the study.
3.3.3 Linearity	**Validation** Analysis of BIT Three calibration curves were established from independently weighed stock solutions. The regression coefficients were 0.9973, 0.9938 and 0.9928.
3.4 Specificity:	**Validation**

Section A4 Analytical Methods for Detection and Identification

Subsection A4.1/3

Annex Point IIA, IV 4.1 (a)

ANALYTICAL METHOD FOR THE DETERMINATION OF 1,2-BENZISOTHIAZOL-3-(2*H*)-ONE IN THE ACTIVE SUBSTANCE AS MANUFACTURED

BIT

	interfering substances	The identity of BIT was confirmed in this test solution by comparison to reference solution. No co-eluting interferences were observed and therefore analysis at this wavelength is also considered to be specific for BIT.
3.5	**Recovery rates at different levels**	**Validation** The accuracy of the assay for the quantification of [redacted] BIT was determined by application of the same procedure to a reference substance of known purity.
3.5.1	Relative standard deviation	**Validation** <u>Analysis of BIT</u> Refer to the relative standard deviation calculated for BIT in the five batch characterisation. (Refer to the Confidential File, Section IIIA 4.1(3) where this summary is presented in full).
3.6	**Limit of determination**	**Validation** <u>Analysis of BIT</u> Limit of Determination: The method is not intended for quantification of trace amounts of [redacted] BIT. Samples will be diluted to fall within the concentration range (1.7 to 2.5 mg/L) covered by the validation. Limit of Detection: The method was not optimised for sensitivity and therefore the lower limit of detection was not established. However the limit of detection was estimated as ≤ 0.008 area-% from a chromatogram of [redacted] BIT.
3.7	**Precision**	
3.7.1	Repeatability	**Validation** <u>Analysis of BIT</u> The precision of the assay with respect to active ingredient content was evaluated by injecting the sample batch solution 10 times and the relative standard deviation of area percent and of area counts was calculated. Acceptable precision was demonstrated..

	Area-(%)	**Area Counts**
RSD (%)	0.00	1.46

3.7.2	Independent	Not applicable

Section A4 Analytical Methods for Detection and Identification

Subsection A4.1/3

Annex Point IIA, IV 4.1 (a)

ANALYTICAL METHOD FOR THE DETERMINATION OF 1,2-BENZISOTHIAZOL-3-(2*H*)-ONE IN THE ACTIVE SUBSTANCE AS MANUFACTURED

BIT

	laboratory validation	
		4 APPLICANT'S SUMMARY AND CONCLUSION
4.1	**Materials and methods**	**Materials and Methods** The aim of this study was to validate an analytical method for the determination of BIT The method validation was performed to demonstrate accuracy, precision, repeatability, linearity and specificity of the analytical method. BIT was determined by HPLC with UV detection and quantification was performed at 350 nm for BIT. **Summary of Results** **Method Validation** Analysis of BIT The analytical method for the assay of [redacted] BIT was successfully validated with respect to accuracy, precision (repeatability, 10 replicate injections of one sample), linearity, specificity (active ingredient and three potential impurities), stability of stock solutions, ruggedness (intermediate precision), limit of determination and limit of detection.
4.2	**Conclusion**	The analytical method for the assay of [redacted] BIT was successfully validated with respect to accuracy, precision, linearity, specificity, stability of stock solutions, ruggedness, limit of determination and limit of detection.
4.2.1	Reliability	1
4.2.2	Deficiencies	No. The study meets the criteria of EPA OPPTS 830.1700 and 830.1800.

Evaluation by Competent Authorities

EVALUATION BY RAPPORTEUR MEMBER STATE

Date	*December 2007*
Materials and methods	*The applicant's version is adopted.*

Section A4 Analytical Methods for Detection and Identification

Subsection A4.1/3

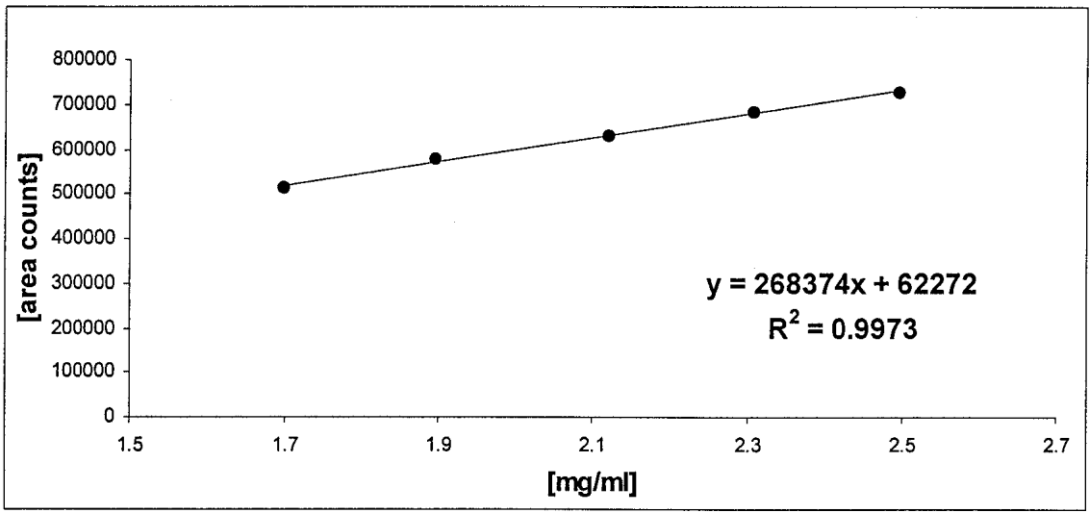
Annex Point IIA, IV 4.1 (a)

ANALYTICAL METHOD FOR THE DETERMINATION OF 1,2-BENZISOTHIAZOL-3-(2*H*)-ONE IN THE ACTIVE SUBSTANCE AS MANUFACTURED

BIT

Conclusion	*The applicant's version is adopted.*
Reliability	*1*
Acceptability	*The method and result are acceptable.*
Remarks	*No further remarks.*

Figure A4_l(3)-1: Typical BIT Calibration Data from Analysis by HPLC with UV Detection at 350 nm



Nominal Concentration (mg/ml)	Measured Concentration (mg/mL)	Relative Derivation (%)	Regression Coefficient R^2
1.697	1.678	-1.0	0.9973
1.896	1.918	1.0	
2.121	2.120	0.0	
2.308	2.318	0.4	
2.495	2.482	-0.5	

Figure A4_1(3)-2: HPLC Chromatogram of a 1.896 mg/mL BIT Calibration Standard with UV Detection at 350 nm

D-7000 HSM: 701763 Series: 0046_001 Report: modified System: Sys 1

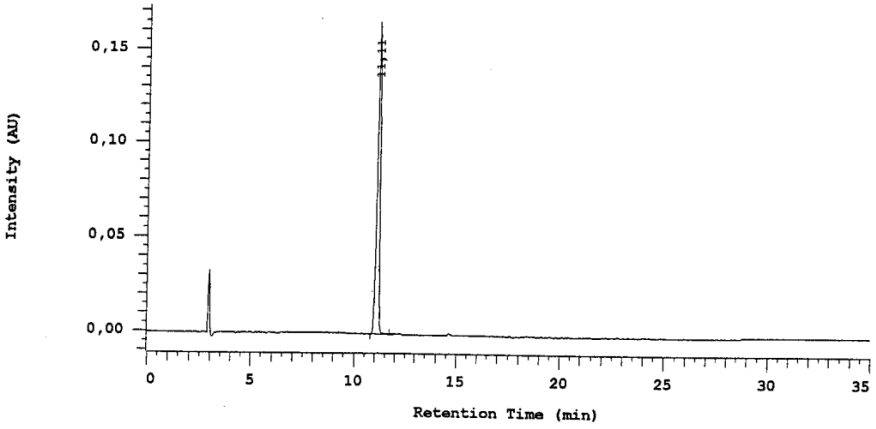
D-7000 HPLC System Manager Report

Analyzed: 08.10.98 16:55
Reported: 09.10.98 07:53
Processed: 09.10.98 07:51

Data Path: C:\WIN32APP\HSM\m1\701763\DATA\0046_001\
Processing Method: [redacted]ExtStd
System(acquisition): Sys 1
Application: 701763
Sample Name: Std. 1.8962 mg/ml
Injection from this vial: 1 of 1
Sample Description:

Series:0046_001
Vial Number: 2
Vial Type: STD2
Volume: 10,0 ul

Chrom Type: Fixed WL Chromatogram, 350 nm



Acquisition Method: [redacted]ExtStd
Column Type: LiChrospher100 RP18e Developed by: OMA
Pump A Type: L-7100
Solvent A: 0.05%H3PO4
Method Description:

Chrom Type: Fixed WL Chromatogram, 350 nm

Peak Quantitation: AREA
Calculation Method: EXT-STD

No.	RT	Name	Area	Height	Conc 1 mg/mL	Purity
1	11,11	[redacted]	577067	82441	1,8962	0,9956
			577067	82441	1,8962	

Peak rejection level: 2000

Section A4 **Subsection A(4.1-4.3)** **Annex Point IIA4.1/4.2 & IIIA-IV.1**	**Analytical Methods for Detection and Identification** **- 4.2 (a) ANALYTICAL METHODS IN SOIL**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ] **Limited exposure [X]**	**Technically not feasible [ ]** **Scientifically unjustified [X]** **Other justification [ ]**	
Detailed justification:	The data requirement for an analytical method in soil indicates that analytical methods in all relevant environmental media are to be provided. In Product Types 6, 13, 9, 11 and 12 there are no direct emission to soil, excluding pesticide use for PT 6, which is covered under other legislation. However, from the various uses, it is possible that indirect exposure to the terrestrial compartment may occur. In order to provide an indication on whether this exposure presents a real risk, it is appropriate to review the derived Risk Characterisation Ratios (RCRs) for the terrestrial compartment. These would indicate the following; PT 6: Max RCR: 4.4×10^{-2}, Min RCR 3.3×10^{-4} PT 13: Max RCR: 2.2×10^{-3}, Min RCR 2.0×10^{-5} Therefore, on the basis of potential exposure, there is limited risk to terrestrial organisms, and therefore limited justification to provide an analytical method for monitoring for BIT. Existing data on the fate of BIT in soil indicates that it is rapidly degraded (t½ = 7.2 hours) in the terrestrial compartment, and that any formed metabolites are transient, and of significantly lower toxicological concern. The SANCO guidance document on residue analytical methods (SANCO/825/00 rev. 7) states that analytical methods are not required for residues in soil where the DT_{90} values of the active substance and relevant metabolites are lower than 3 days. The DT_{90} for BIT is 24 hours, and there are no metabolites of toxicological concern (as discussed in the Doc. II-A). As well as emphasising the very low risk to the terrestrial environment, the development of analytical methods for rapidly degraded molecules is technically challenging. The SANCO guidance recognises the residue cut-off limit of 0.05 mg/kg wwt as a target LOQ in soil. The highest modelled concentration for BIT in all of the use scenarios is 0.0013 mg/kg wwt, well below the level which SANCO recognises as technically feasible. Therefore it is considered that the very low risk posed to the terrestrial environment does not justify the considerable effort which would be required to provide a fully validated method in soil. Therefore a justification for non-submission of data is proposed on the basis of	

Section A4 **Subsection A(4.1-4.3)** **Annex Point IIA4.1/4.2 & IIIA-IV.1**	**Analytical Methods for Detection and Identification** **- 4.2 (a) ANALYTICAL METHODS IN SOIL**
	1. Limited exposure 2. Scientifically not justified
Undertaking of intended data submission [ ]	
	Evaluation by Competent Authorities
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*December 2007.*
Evaluation of applicant's justification	*The non-submittion of data is justified.*
Conclusion	*Acceptable.*
Remarks	*No further remarks.*

Section A4 **Subsection A(4.1-4.3)** **Annex Point IIA4.1/4.2 & IIIA-IV.1**	**Analytical Methods for Detection and Identification** **- 4.2 (b) ANALYTICAL METHODS IN AIR**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ] **Limited exposure [X]**	**Technically not feasible [ ]** **Scientifically unjustified [ ]** **Other justification [ ]**	
Detailed justification:	The data requirement for Analytical Methods for Detection and Identification indicates that analytical methods in all relevant environmental media are to be provided. The data requirement for an analytical method in air indicates that analytical methods in air need to be submitted e.g. if the substance is volatile (i.e. if the vapour pressure > 0.01 Pa) or sprayed, or occurrence in air is otherwise probable. BIT has the following relevant physical properties; 20°C 6.3×10^{-5} Pa 25°C 1.4×10^{-4} Pa 30°C 3.1×10^{-4} Pa TGAS (73.6%) 3.7×10^{-4} Pa Henry's law constant (calculated) 2.3×10^{-9} Pa m^3/mol The Technical Grade Active Substance is supplied as a wet paste, which precludes the possibility of spontaneous dust cloud generation. In Product Types 6, 13, 9, 11 and 12 emissions to air are expected to be negligible. The product is supplied as a 20% solution in glycol, therefore evaporation into air is not considered probable. In downstream products (PT 6), there is only one scenario whereby Technical Grade BIT may be included in preparations which are to be applied in a manner which generates aerosols, particles or droplets. The application system is the "trigger spray" type, which, according to RIVM report 320104003 (Table 9, page 29), has MMAD values ranging from 63-133 µm for all trigger sprays, with the MMAD for an all purpose cleaner (the largest market penetration) recorded as 133 µm. This droplet size is not considered to be inhalable, therefore exposure through the limited occurrence in air is not probable. Therefore a justification for non-submission of data is proposed on the basis of Limited exposure	
Undertaking of intended data submission [ ]		

Section A4 **Subsection A(4.1-4.3)** **Annex Point IIA4.1/4.2 & IIIA-IV.1**	**Analytical Methods for Detection and Identification** **- 4.2 (b) ANALYTICAL METHODS IN AIR**
	Evaluation by Competent Authorities
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*July 2010.*
Evaluation of applicant's justification	*The non-submittion of data is justified.*
Conclusion	*Acceptable.*
Remarks	*No further remarks.*

Section A4 Analytical Methods for Detection and Identification

Subsection A4.2c ANALYTICAL METHOD FOR THE DETERMINATION OF ACTIVE SUBSTANCE RESIDUES IN WATER

Annex Point IIA, IV4.1/4.2 & IIIA-IV.1

			Official use only
		1 REFERENCE	
1.1	**Reference**	[redacted] 2007; Validation of a residue analytical method for the determination of 1,2-Benzisothiazol-3-(2*H*)-one in drinking water; [redacted] Study No. B46620; GLP; Unpublished.	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Arch Chemicals Inc, Clariant Production UK Ltd and Thor GmbH	
1.2.2	Companies with letter of access	Not applicable	
1.2.3	Criteria for data protection	Data on existing substance for first entry in to Annex I.	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	Yes SANCO/825/00 Revision 7	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Preliminary treatment**		
3.1.1	Enrichment	10 mL aliquots of drinking water (Refer to Table 4.2(c)-1 for the water characteristics) were transferred into glass vessels and fortified as appropriate. Methanol (1 mL) was added and the sample was mixed well prior to analysis by LC-MS/MS.	
3.1.2	Cleanup	Not applicable	
3.2	**Detection**		

Section A4 Analytical Methods for Detection and Identification

Subsection A4.2c ANALYTICAL METHOD FOR THE DETERMINATION OF ACTIVE SUBSTANCE RESIDUES IN WATER

Annex Point IIA, IV4.1/4.2 & IIIA-IV.1

3.2.1 Separation method

HPLC Conditions and Instrument:

Column: Zorbax SB Phenyl; 5 µm, (50 mm x 2.1 mm)

Injection Volume: 50 µL

Autosampler: CTC PAL

Pumps: Shimadzu LC l0AD

Eluent A: 5 mmol/L ammonium formate and 0.1% formic acid in methanol/water (5+95; v/v)

Eluent B: 5 mmol/L ammonium formate and 0.1% formic acid in methanol/water (95+5; v/v)

Flow Rate: 300 µL/minute

Gradient:

Time (min)	**0**	**3.0**	**3.1**	**3.5**	**3.6**	**5.0**
A(%)	80	40	0	0	80	80
B(%)	20	60	100	100	20	20

Typical Retention Time: 2.5 minutes

3.2.2 Detector

Detection: Tandem mass spectrometry with electrospray ionisation in positive mode.

Instrument: Sciex API 5000 using ANALYST software

Conditions:

Nebuliser Gas: Air

Heater Gas: Air

Curtain Gas: Nitrogen

Collision Gas: Nitrogen

Ionisation Mode: pneumatically and thermally assisted electrospray ionisation (ESI)

Ion Source: Sciex Turbo-V-Source

Heater Gas Temperature: 500°C

Spray Voltage: 2500 V

Scan Mode: Multiple Reaction Monitoring (MRM)

	Ion	**Precursor**	**Product**	**Collision**

Section A4 Analytical Methods for Detection and Identification

Subsection A4.2c ANALYTICAL METHOD FOR THE DETERMINATION OF ACTIVE SUBSTANCE RESIDUES IN WATER

Annex Point IIA, IV4.1/4.2 & IIIA-IV.1

	Polarity	Ion (m/z)	Ion (m/z)	Energy (V)
Transition 1 (Primary method : used for Quantification)	Positive	152	105	33
Transition 2 (confirmatory method)	Positive	152	109	32

3.2.3 Standard(s)

External standard: BIT with a purity of > 99% and an expiry date of 28 February 2008.

Calibration Standards

The BIT analytical standard detailed above was used to prepare the calibration line. Six calibration standards ranging from 0.025 ng/mL to 1.0 ng/mL were prepared in methanol/water (1 + 9; v/v).

Fortification Solutions

The BIT analytical standard detailed above was also used to prepare fortification solutions of 0.1 µg/mL and 0.01 µg/mL in methanol.

3.2.4 Interfering substance(s)

Due to the selective nature of the LC-MS/MS method of analysis, there are no substances expected to interfere with BIT.

3.3 Linearity

3.3.1 Calibration range

0.025 to 1.0 ng/mL

3.3.2 Number of measurements

Six calibration standards in the range of 0.025 to 1.0 ng/mL.

3.3.3 Linearity

correlation coefficient (r^2) = 1.000

The correlation was calculated using a least square fit of a linear function.

Data from the calibration lines generated from the MS/MS transition used for quantitation (primary method) and confirmation are summarised below:

Range (ng/mL)	Intercept (a)	Slope (b)	Correlation Coefficient

Section A4 Analytical Methods for Detection and Identification

Subsection A4.2c ANALYTICAL METHOD FOR THE DETERMINATION OF ACTIVE SUBSTANCE RESIDUES IN WATER

Annex Point IIA, IV4.1/4.2 & IIIA-IV.1

			(r^2)
Primary Method			
0.025 – 1.0	107	181785	1.000
Confirmatory Method			
0.025 – 1.0	617	256801	1.000

Regression Model: y = a + b * X

Refer to Figure 4.2(c)-1 for a representative chromatogram (primary and confirmatory method) of a 0.025 ng/mL calibration standard.

3.4 Specificity: interfering substances

There was no detectable signal found in either of the two control samples at the retention time of BIT.

No interferences were found at the retention time of BIT above 30% of the limit of quantification as well as above the limit of detection.

Therefore the method was found to be specific for the determination of BIT in drinking water.

Refer to Figure 4.2(c)-2 for a representative chromatogram (primary and confirmatory method) of a control drinking water sample.

3.5 Recovery rates at different levels

Fortification Level (µg/L)	Range	Mean Recovery (%)	Coefficient of Variation (%)	Number of Analyses
Primary Method				
0.1	104-108	106	1	5
1.0	106-115	110	3	5
Overall	104-115	108	3	10
Confirmatory Method				
0.1	97-107	101	4	5
1.0	105-116	110	4	5
Overall	97-116	106	6	10

Refer to Figure 4.2(c)-3 for a representative chromatogram (primary and

confirmatory method) of a control drinking water sample fortified with BIT at 0.1 µg/L.

3.5.1 Relative standard deviation

Fortification Level (µg/L)	Relative Standard Deviation (Coefficient of Variation, %)
0.1	**1**
1.0	**3**
Overall	**3**
0.1	**4**
1.0	**4**
Overall	**6**

3.6 Limit of determination

The limit of detection (LOD) was estimated from the lowest calibration standard concentration used (0.025 ng/mL).

The corresponding limit of detection for BIT in drinking water is 0.028 µg/L.

The validated limit of quantification is 0.1 µg/L for BIT in drinking water.

3.7 Precision

3.7.1 Repeatability

Primary Method

The relative standard deviations (coefficient of variation) achieved at 0.1 µg/L (limit of quantification) and 1.0 µg/L were 1% and 3%, respectively. The data demonstrate the precision (repeatability) of the analytical method for BIT in drinking water.

Confirmatory Method

The relative standard deviation (coefficient of variation) achieved at 0.1 µg/L (limit of quantification) and 1.0 µg/L was 4%. The data demonstrate the precision (repeatability) of the analytical method for BIT in drinking water.

3.7.2 Independent laboratory validation

Not applicable

4 APPLICANT'S SUMMARY AND CONCLUSION

Section A4 Analytical Methods for Detection and Identification

Subsection A4.2c

ANALYTICAL METHOD FOR THE DETERMINATION OF ACTIVE SUBSTANCE RESIDUES IN WATER

Annex Point IIA, IV4.1/4.2 & IIIA-IV.1

4.1	**Materials and methods**	Local tap water from Schopfheim, Germany was sampled and characterised prior to analysis. An analytical method for the determination of BIT in drinking water was validated. 5 replicates at 0.1 and 1.0 µg/L; and control samples were prepared and analysed by HPLC-MS/MS employing electrospray in positive ionisation mode. 10 mL aliquots of the water samples were transferred to glass vessels and fortified as appropriate. Methanol (1 mL) was added and the sample was mixed well prior to analysis by LC-MS/MS. Two MS/MS mass transitions were monitored. The data from both the primary and confirmatory transitions were acceptable in terms of accuracy, precision, specificity and linearity. The method limit of detection was 0.025 ng/mL with a corresponding limit of detection for BIT in drinking water of 0.028 µg/L. The limit of quantification for the validated method was 0.1 µg/L.
4.2	**Conclusion**	A method with a limit of quantification of 0.1 µg/L was validated for the determination of BIT in drinking water. The LC-MS/MS method was found to be acceptable in terms of accuracy, precision, specificity and linearity. Therefore the sampling and analytical techniques are considered to be suitable as a monitoring method for BIT in drinking water.
4.2.1	Reliability	1
4.2.2	Deficiencies	No

Evaluation by Competent Authorities

EVALUATION BY RAPPORTEUR MEMBER STATE

Date	*December 2007.*
Materials and methods	*The applicant's version is adopted*
Conclusion	*The applicant's version is adopted*
Reliability	*1*
Acceptability	*The method and result are acceptable*

Section A4	**Analytical Methods for Detection and Identification**
Subsection A4.2c **Annex Point IIA, IV4.1/4.2 & IIIA-IV.1**	**ANALYTICAL METHOD FOR THE DETERMINATION OF ACTIVE SUBSTANCE RESIDUES IN WATER**
Remarks	*No further remarks*

Table 4.2(c)-1: Drinking Water Characteristics

Source of Drinking Water	Local Tap Water from Schopfheim, Germany. Collected 30 May 2007.
Dry Residue	85.7 mg/L
Silt Content	0.1 mg
pH-Value	7.98
Dissolved Organic Carbon	0.61 mg/L
Hardness	5°dH

Figure 4.2(c)-1: Chromatogram Obtained from LC-MS/MS Analysis (Primary and Confirmatory Method) of a 0.025 ng/mL Calibration Solution.

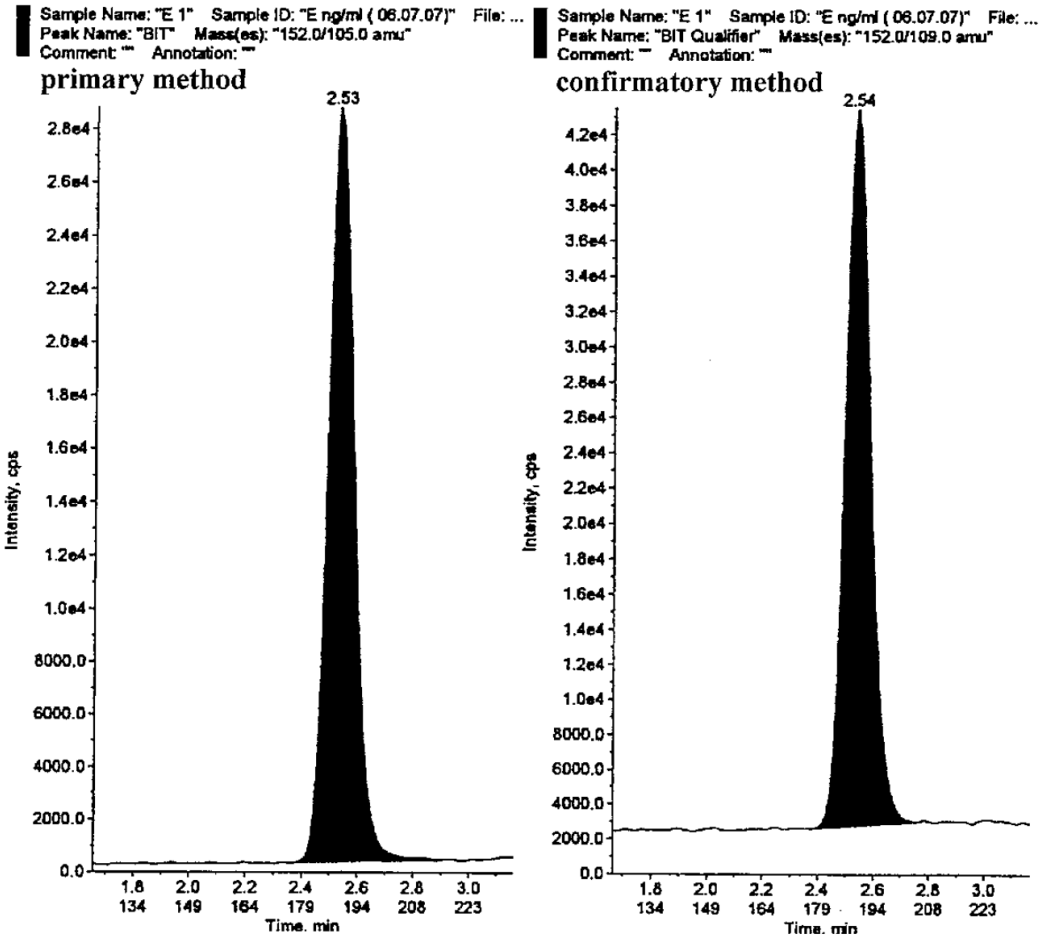

Sample Name: "E 1" Sample ID: "E ng/ml (06.07.07)" File: ...
Peak Name: "BIT" Mass(es): "152.0/105.0 amu"
Comment: "" Annotation: ""
primary method
2.53
Intensity, cps
Time, min
Sample Name: "E 1" Sample ID: "E ng/ml (06.07.07)" File: ...
Peak Name: "BIT Qualifier" Mass(es): "152.0/109.0 amu"
Comment: "" Annotation: ""
confirmatory method
2.54
Intensity, cps
Time, min

Figure 4.2(c)-2: Chromatogram Obtained from LC-MS/MS Analysis (Primary and Confirmatory Method) of a Control Drinking Water Sample

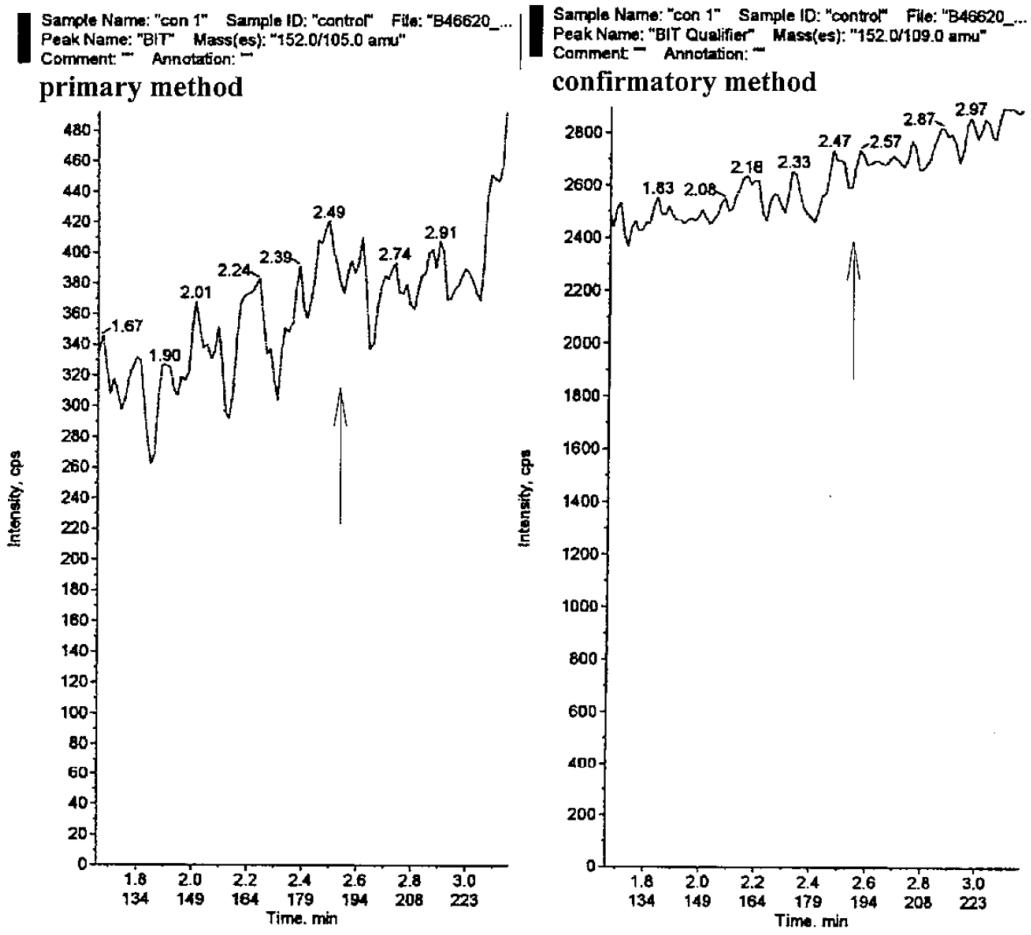
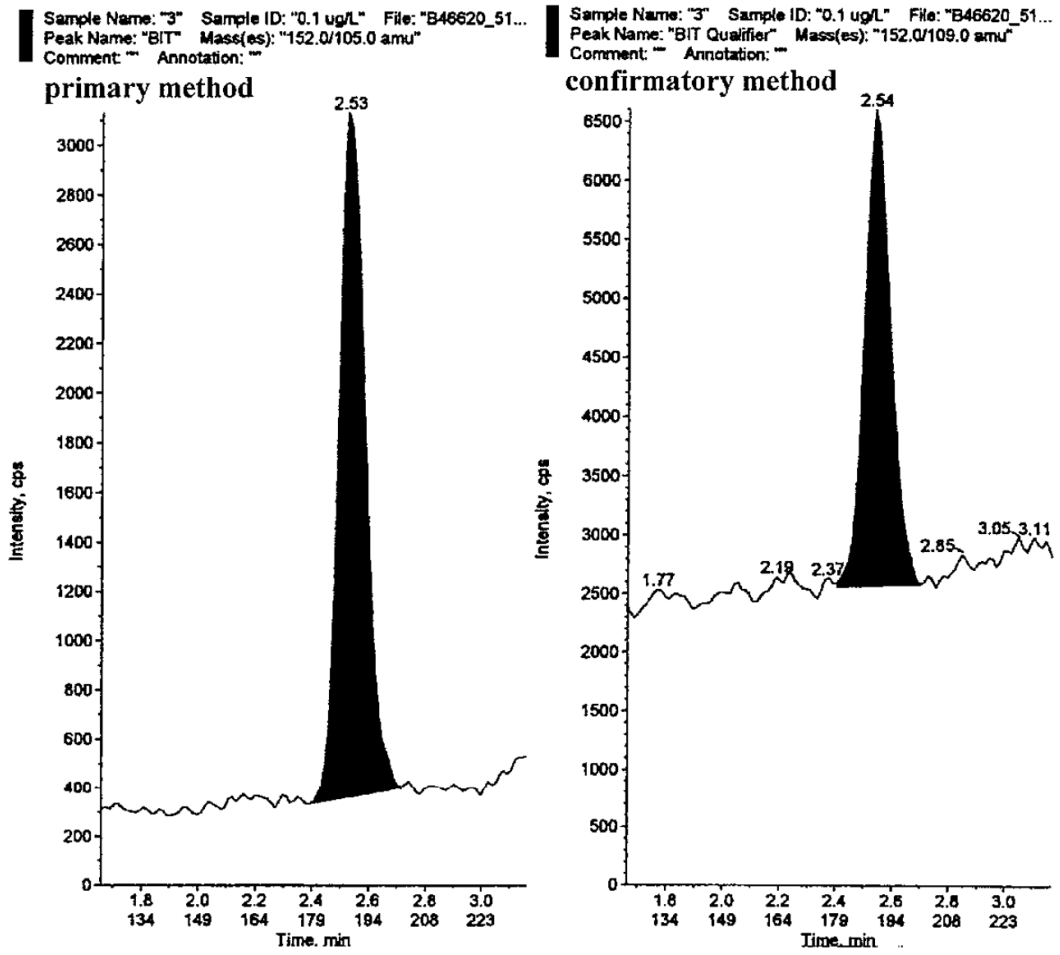


Figure 4.2(c)-3: Chromatogram Obtained from LC-MS/MS Analysis (Primary and Confirmatory Method) of a Control Drinking Water Sample Fortified at 0.1 µg/L



Section A4 **Subsection A(4.1-4.3)** **Annex Point IIA, IV4.1/4.2 & IIIA-IV.1**	**Analytical Methods for Detection and Identification** **- 4.2 (d) ANALYTICAL METHODS IN ANIMAL AND HUMAN BODY FLUIDS AND TISSUES**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ] **Limited exposure [ ]**	**Technically not feasible [ ]** **Scientifically unjustified [ ]** **Other justification [X]**	
Detailed justification:	The data requirement for Analytical Methods for Detection and Identification indicates that analytical methods in all relevant environmental media are to be provided. The data requirement for an analytical method in animal and human body fluids and tissues indicates that analytical methods need to be submitted where an active substance is classified as toxic or highly toxic. BIT is not classified as toxic or highly toxic, therefore the requirement criteria for this endpoint are not met. Therefore a justification for non-submission of data is proposed on the basis of BIT not fulfilling the toxicity criteria required to trigger this data point.	
Undertaking of intended data submission [ ]		
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*December 2007.*	
Evaluation of applicant's justification	*The non-submission of data is justified*	
Conclusion	*Acceptable*	
Remarks	*No further remarks*	

Section A4 **Subsection A(4.1-4.3)** **Annex Point IIA, IV4.1/4.3 & IIIA-IV.1**	**Analytical Methods for Detection and Identification** **- 4.3 ANALYTICAL METHODS INCLUDING RECOVERY RATES AND THE LIMITS OF DETERMINATION FOR RESIDUES IN/ON FOOD OR FEEDSTUFFS AND OTHER PRODUCTS WHERE RELEVANT**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ] **Limited exposure [X]**	**Technically not feasible [ ]** **Scientifically unjustified [ ]** **Other justification [ ]**	
Detailed justification:	The data requirement for Analytical Methods for Detection and Identification indicates that analytical methods in all relevant environmental media are to be provided. Analytical methods including recovery rates and the limits of determination for residues in/on food or feedstuffs and other products where relevant are required if the active substance or the material treated with it is to be used in a manner which may cause contact with food or feedstuffs. An assessment of the uses of BIT in Product Types 6, 13, 9, 11 and 12 being supported in this dossier concludes that this requirement is not triggered. Therefore a justification for non-submission of data is proposed on the basis of BIT not fulfilling the exposure criteria required to trigger this data point.	
Undertaking of intended data submission [ ]		
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*December 2007.*	
Evaluation of applicant's justification	*The non-submission of data is justified*	
Conclusion	*Acceptable*	
Remarks	*No further remarks*	

Section A5 Effectiveness against target organisms and intended uses

Subsection (Annex Point)			Official use only
5.1	**Function (IIA5.1)**	Main Group 2: Preservatives PT13 Metal-working fluid preservatives: bactericide/fungicide	
5.2	**Organism(s) to be controlled and products, organisms or objects to be protected (IIA5.2)**	-	
5.2.1	Organism(s) to be controlled (IIA5.2)	Typical microbes to be controlled Bacteria, fungi and yeast including but not exclusively	

Bacteria

- *Bacillus subtilis*
- *Burkholderia cepacia*
- *Enterobacter cloacae*
- *Escherichia coli*
- *Proteus vulgaris*
- *Pseudomonas aeruginosa*
- *Streptococcus lactis*
- *Pseudomonas putida*
- *Staphylococcus aureus*
- *Streptococcus lactis*

Fungi

- *Alternaria alternata*
- *Aspergillus niger*
- *Aureobasidium pullulans*
- *Chaetomium globosum*
- *Chladosporium cladosporoides*
- *Penicillium notatm*
- *Fusarium solani*
- *Fusarium oxysporum*

Yeast

- *Candida albicans*
- *Rhodotorula rubra*
- *Saccharomyces cerevisiae*

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5.2.2	Products, organisms or objects to be protected (IIA5.2)	BIT is placed on the market as a 20% water miscible preparation in dipropylene glycol ("20% BIT/GLYCOL"). PT13: metal-working fluid preservation.	X
5.3	**Effects on target organisms, and likely concentration at which the active substance will be used (IIA5.3)**		
5.3.1	Effects on target organisms (IIA5.3)	Effect on target organism: BIT has an antimicrobial action. The interaction of BIT with bacteria, fungus or yeast is influenced by the metabolic activity of the organisms (this has been demonstrated in the Gram-positive bacteria *Staphylococcus aureus*). Effect of concentration: The antimicrobial of activity of BIT increases at higher concentrations of BIT. The optimal concentration of BIT should be determined for each use. Refer to Section 5.3.2 for the likely concentrations for each use.	
5.3.2	Likely concentrations at which the A.S. will be used (IIA5.3)		
	PT13	PT13 Metal-working fluid preservatives i) Metal Working Fluid Concentrate The most effective concentration of "20% BIT/GLYCOL" for a metalworking fluid concentrate is 2-3%, product as supplied (approximately 0.4-0.6% or 4000-6000 ppm as active BIT). This treatment level for the metalworking fluid is based on two factors, consideration of the final end-use dilution of the coolant and the effective dose for BIT against the target organisms typically found in this application. ii) Metal Working Tank Additive For tank-side use a dose of 1000-1500 ppm "20% BIT/GLYCOL" (approximately 0.02 to 0.03% or 200-300 ppm active BIT) would be added to the dilute coolant into an area of good agitation.	

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5.4	**Mode of action (including time delay) (IIA5.4)**	-	
5.4.1	Mode of action	BIT inhibits the oxidation of a number of carbohydrate substrates, which are transported across the cytoplasmic membrane by a process involving thiol dependent enzymes. In addition, BIT inhibits the oxidation of glycerol, which enters the cell by diffusion and also inhibits the utilisation of the electron transport chain by bacteria - possibly by action on the dehydrogenase enzymes. The inhibition of a number of these thiol-containing enzymes isolated from *Staphylococcus aureus* supports this hypothesis. The interaction of BIT with the Gram- positive bacteria *Staphylococcus aureus* is influenced by the metabolic activity of the organisms. Enzymes dependent upon thiol groups for activity are affected by BIT and consequently inhibition of metabolic processes involving these enzymes is observed. A mechanism of antibacterial action such as this is unlikely to prove rapidly bactericidal; and is supported by the preservative rather than the disinfective action of BIT. **References:** [redacted] Antimicrobial - 1,2-Benzisothiazolin-3-one (BIT) - A Review of the Mechanism of Bacterial Action – ARCH Technical Information Bulletin (2005).	
5.4.2	Time delay	No time delay.	
5.5	**Field of use envisaged (IIA5.5)**		
	MG02: Preservatives	PT13 Metal-working fluid preservatives • Metal working fluid concentrate • Metal working tank additive	
5.6	**User (IIA5.6)**		
	Industrial	**PT13 Metal-working fluid preservatives** <u>Metal working fluid concentrate</u> Personnel involved in the manufacturing of the metalworking fluid (formulator). Personnel at the end-user site (parts manufacturer) who would add the biocide into the dilute coolant (already in the system). Personnel at the end-user site (end-user) who would use the preserved metalworking fluid.	**X1**

Section A5 Effectiveness against target organisms and intended uses

	Professional	**PT13 Metal-working fluid preservatives** Professional use is not applicable for this product type.	**X2**
	General public	**PT13 Metal-working fluid preservatives** Use by the general public is not applicable for this product type.	
5.7	**Information on the occurrence or possible occurrence of the development of resistance and appropriate management strategies** **(IIA5.7)**		
5.7.1	Development of resistance	BIT, the active ingredient in [redacted] biocides derives its antimicrobial activity from its ability to react with thiol compounds. Thiol compounds are found in cell membranes and serve important roles in various crucial metabolic activities such as transport of essential nutrients and minerals, respiration, excretion of waste products and many other membrane related processes. This mode of attack is fairly general and cannot be overcome by cells. Any possible method to overcome the ability of BIT to react with cell membranes has consequences that lead to slowing down of metabolism and contributes to eventual cell death. The thiol groups, found in many crucial enzymes responsible for driving many cellular processes, are also inhibited by BIT. Thus BIT has a principal mode of attack followed by a secondary inhibition of vital enzymes. This multiple attack mode precludes the possibility for organisms to develop mechanisms that can be passed on to future generations in the form of "resistance". **Reference:** [redacted] Not GLP; Unpublished.	
5.7.2	Management strategies	Formulators and manufacturers of the products requiring protection should adopt the following management strategies to avoid microbial spoilage: • Select the appropriate biocide for the product by consulting biocides professionals. • Eliminate conditions that could lead to biocide incompatibility. • Use optimised levels of biocides, determined by standard challenge tests.	

Section A5 Effectiveness against target organisms and intended uses

- Follow good hygiene practices at the production facility.
- Avoid the presence of redox active agents, unreacted monomers of polymerisation reactions and other active chemical species.
- Avoid situations that could lead to formation of biofilms (Individual bacteria, when in contact with a solid surface, organize in to a community that behaves as a single larger organism. Often the amount of biocides required to kill these communities is much higher than the minimum ihibitory concentration that are seen for the same organisms before they form these films).

5.8	**Likely tonnage to be placed on the market per year (IIA5.8)**	Refer to Confidential File, Section IIIA 5.

Evaluation by Competent Authorities

EVALUATION BY RAPPORTEUR MEMBER STATE

Date

October 2011.

March 2015

May 2020

Materials and Methods

The table 5.3: Summary table of experimental data on the effectiveness of BIT against target organisms includes the summaries of the studies but they are not included in Doc IIIA format.

The other components included in the composition (solvents) do not affect to the efficacy of the active substance BIT.

[redacted] *1995: the study contains efficacy data on one product with 20% BIT as an industrial preservative. The data is divided into two sections. The first section deals with assessing anti-microbial efficacy in a variety of industrial matrixes. The second section deals with determination of the minimum concentration of one product required to inhibit the growth of specific organisms in culture media. The study demonstrated efficacy of the active substance. Considering the worst case, the study demonstrates bactericidal activity at 0.005%BIT (250ppm of the biocidal product acts against P. aeruginosa) and fungicidal activity at 0.012%BIT (600ppm of the biocidal product acts against A. penicilloides)*

This study was evaluated under PT6 too.

Section A5 Effectiveness against target organisms and intended uses

	[redacted] *1992: the study provides supplemental data on the efficacy of one product with 20%. The study demonstrated efficacy of the active substance for other bacteria and fungi. The concentration of BIT necessary to acts against these organisms is less than the study* [redacted]*.; 1991. The study* [redacted] *1991 is considered worst case.* [redacted] *1994: the study provides efficacy data on one product with 20% BIT. Recovery tests were conducted and showed that the product is effective in recovering spoiled or contaminated metalworking fluid systems.* *This study was evaluated under PT13 too.* *May 2020* *All the unpreserved controls showed growth.* *Summaries of the studies in tabular DocIII format were requested from the applicant.* *December 2020:* *The applicant submitted the summaries of the studies in tabular DocIII format on December 2020.*
Conclusion	*The efficacy tests are in Doc. IV-A but no in Doc. III-A (the applicant has included the studies in the table 5.3.1 only). We do not have the summary studies in Doc. III-A:* *Doc. IV-A 5.3.1_2: the study contains efficacy data on one product with 20% BIT as an industrial preservative. The data is divided into two sections. The first section deals with assessing anti-microbial efficacy in a variety of industrial matrixes. The second section deals with determination of the minimum concentration of one product required to inhibit the growth of specific organisms in culture media. The study demonstrated efficacy of the active substance.* *Doc. IV 5.3.1_6: the study provides supplemental data on the efficacy of one product with 20%. The study demonstrated efficacy of the active substance.* *Doc. IV 5.3.1_7: the study provides efficacy data on one product with 20% BIT. Recovery tests were conducted and showed that the product is effective in recovering spoiled or contaminated metalworking fluid systems.* *The information included in the dossier was considered acceptable. The innate efficacy of BIT was demonstrated against bacteria and fungi. Nevertheless, further efficacy data will be required to support the authorisation at the member state level.*
Reliability	*2*

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Acceptability	*The studies about efficacy and information about development of resistance and management strategies to avoid bacterial growth and spoilage were considered acceptable* *Acceptable*
Remarks	***X1*** *Indirect exposure of operators to metal parts contaminated with preserved metal working fluid during transport of machined pieces to storage is addressed.* *Industrial exposure is considered for operators working in the formulation of biocidal product into metal working fluid concentrate.* ***X2*** *Professional exposure is considered for operators working in metalworking premises.* *We accept the information included in this section considering the following remarks.* *X1: The "Transitional Guidance on Efficacy Assessment for preservatives" was not published when the dossier was submitted.* *X: the active substance is active against bacteria and fungi*

Table A5.1: Summary table of experimental data on the effectiveness of the active substance against target organisms at different fields of use envisaged, where applicable

PT13

Function	Field of Use Envisaged	Test Substance	Test Organism(s)	Test Method (Refer to Table 5.3(2) for Description of Test Method)	Test Conditions	Test Results: Effects, Mode of action, resistance	Reference
MG2 PT13	Metal Working Fluid Sample G165	[redacted]	Natural isolate from spoiled product	Recovery Test[1]	Concentration: Unpreserved, 50, 80, 100, 250, 500, 1000 and 2000 ppm	Result: Efficacy at 500 ppm (0.05%)	[redacted] 1994
MG2 PT13	Metal Working Fluid-Microgrind 573-PTC	[redacted]	Natural isolate from spoiled product (1.56×10^3viable bacteria/ml) and 10% Standard Laboratory Inoculum	Recovery Test[1] (9 Days Post Inoculation)	Concentration: Unpreserved, 500, 750, 1000, 1250, 1500, 2000 and 2500 ppm	Result: Efficacy at 1250 ppm (0.125%)	

MG2 PT13	Steel Rolling Emulsion (Sample B1003-93)	[redacted]	Natural isolate from spoiled product (1.2×10^5 viable bacteria/g and fungal colonies)	Recovery Test[1] (7 Days Post Inoculation) at 110 °F (43.3 °C)	Concentration: Unpreserved, 500, 750, 1000, 1250, 1500, 2000 and 2500 ppm (% [redacted]	Result: Efficacy at 500 ppm (0.05%)	
MG2, PT13	Metal Working Fluid (sample 052394)	[redacted]	Pseudomonas aeruginosa	Challenge Test[2] (4 inoculations)	Concentration: Unpreserved, 0.05, 0.1, 0.2, 0.25 and 0.3 %	Result: Efficacy at 0.05%	[redacted] 1995
	Metal Working Fluid (sample 100694)	[redacted]	Naturally occurring spoilage organisms	Recovery Test[1]	Concentration: Unpreserved, 0.025, 0.05, 0.075, 0.1, 0.15 and 0.20 (% [redacted]	Result: Efficacy at 0.025%	
NA	NA - Bacterial /Fungal Suspension with [redacted] only.	[redacted]	*Pseudomonas aeruginosa* *Escherichia coli* *Klebsiella pneumoniae* *Staphlococcus aureus* *Bacillus subtilis* *Proteus Vulgaris* *Bacillus megaterium* *Salmonelle typhosa* *Aspergillus niger* *Tricophyton mentagrophytes* *Aspergillus penicilloides* *Alternaria radicina* *Rhizopus stolonifer*	Minimum Inhibitory Concentration (MIC) Test[3]	600 to 6.3 ppm and control .	MIC of [redacted] against the selected bacteria: *Pseudomonas aeruginosa: 250.0 ppm* *Escherichia coli: 31.3 ppm* *Klebsiella pneumoniae: 31.3 ppm* *Staphlococcus aureus: 18.8 ppm* *Bacillus subtilis: 7.8 ppm* *Proteus Vulgaris: 9.4 ppm* *Bacillus megaterium: 18.8 ppm* *Salmonelle typhosa: 18.8 ppm* MIC of [redacted] against the selected Fungi: *Aspergillus niger: 200 ppm* *Tricophyton mentagrophytes: 25.0 ppm* *Aspergillus penicilloides: 600.0 ppm* *Alternaria radicina: 150 ppm* *Rhizopus stolonifer: 40.0 ppm*	

MG2, PT13	Metal Working Fluid	[redacted]	*Pseudomonas aeruginosa* *Enterobacter cloacae* *Escherichia coli* *Acinetobacter calcoaceticus*	Recovery Test[1]	Concentration: Unpreserved, 250, 500, 750, 1000, 1250, 1500, 2000, 2500 and 3000 ppm	Result: Efficacy at 1250 ppm (0.125%)	[redacted].; 1992;
NA	NA - Bacterial /Fungal Suspension with [redacted] only.	[redacted]	Bacteria *Bacillus megaterium* *Salmonella tvphi* *Klebsiella pneumoniae* *Fungi* *Aspergillus penicilloides* *Rhizopus stolonifer* *Alternaria radicina* *Trichophyton mentagrophytes*	Minimum Inhibitory Concentration (MIC) Test[4]	Concentration: Unpreserved, 10, 25, 50, 100, 150, 200, 250 and 300 ppm	MIC of [redacted] against selected bacteria, fungi and yeast Bacteria *Bacillus megaterium: 25 ppm* *Salmonella tvphi: 25 ppm* *Klebsiella pneumoniae: 50 ppm* *Fungi* *Aspergillus penicilloides: 50 ppm* *Rhizopus stolonifer: 100 ppm* *Alternaria radicina|: 50 ppm* *Trichophyton mentagrophytes: 50 ppm*	

NA = not applicable

Section 5.3: Table 5.3(2): Description of Test Methods

Test Method and Author	Method Description
Recovery Test[1] Procedure applicable to: [redacted], 1995; [redacted], 1994; [redacted], 1992 (Doc IVA 5.3.1_7)	This test is designed to ascertain the concentration of preservative necessary to eliminate established microbiological contamination in spoiled samples. Aliquots of the sample were dispensed into pre-sterilised containers. Biocide was added to the containers to create samples with increasing biocide concentration. After the addition of biocide the microbiological status of each sample was determined (on a minimum of three occasions) over a period of seven days by streak plating or dilution plate counting of the samples. The plates were incubated for 48 hours at 30°C and then assessed for the degree of microbial contamination. The lowest level of biocide which eliminates all viable growth after the final challenge is considered to be the minimum effective concentration.
Challenge Test[2] Procedure applicable to: [redacted], 1995 (Doc IVA 5.3.1_2)	This test is designed to ascertain the concentration of preservative necessary to prevent microbial growth in a sample when deliberately inoculated with spoilage microorganisms. Aliquots of the product were placed in individual containers and biocide was added to the containers to create a ladder of samples with increasing biocide concentration. The product in each of these containers was challenged at least three times with the appropriate inoculum of common spoilage organisms, in which the bacteria population was at least 1.0 x 10^6 CFU/ml. After each challenge the efficacy of the added preservative is monitored by periodically (on a minimum of three occasions) streaking out the samples onto sterile agar plates. The plates were incubated for 48 hours at 30°C and then assessed for the degree of microbial contamination. The lowest level of biocide which eliminates all viable growth after the final challenge is considered to be the minimum effective concentration.
Minimum Inhibitory Concentration (MIC) Test[1] Procedure applicable to: [redacted], 1995 (Doc IVA 5.3.1_2)	Bacterial and fungal suspensions were prepared as appropriate. [redacted] was incorporated into the bacterial and fungal suspensions and serial dilutions were performed such that the bacterial and fungal suspensions were exposed to a range of [redacted] concentrations (21 concentrations in the range of 6.3 to 600.0 ppm). The samples were incubated at 30 °C for the appropriate time (48 hours for bacteria and 1 week for fungi). Samples were examined for evidence of growth and the results were reviewed to determine the lowest concentration of biocide in which no new growth is observed. The concentration in this sample is considered to be the MIC.
Minimum Inhibitory Concentration (MIC) Test[5] Procedure applicable to: [redacted], 1992 **(**Doc IVA 5.3.1._6)	All cultures were obtained from the American Type Culture Collection. Bacterial inocula were grown in tryptic soy broth at 30 °C. Fungal inocula were grown in Sabourand dextrose broth at ambient temperature. A ladder of concentrations of the respective biocides was made in the same medium used for growth and inoculated with an actively growing culture. The reported MIC is the lowest concentration that yielded no visible growth at 48 hours.

Section A5.3.1/01 Annex Point IIA, V.5.3 **Efficacy Data**

[redacted] MIC data and efficacy data in various products

		Official use only
	1 REFERENCE	
1.1 Reference	[redacted] MIC data and efficacy data in various products. [redacted] 1995	
1.2 Data protection	Yes	
Data owner	Lonza Cologne GmbH	
Companies with letter of access		
Criteria for data protection	Data on existing substance for first entry into Annex I.	
1.3 Guideline study	Products for Processing and Industrial Uses (Guideline Ref. No. 91-54).	
1.4 Deviations	None stated.	
	2 METHOD	
2.1 Test Substance (Biocidal Product)	[redacted]	
Trade name/ proposed trade name		
Composition of Product tested	1,2-benzisothiazol-3(2H)-one at 20% active ingredient	
Physical state and nature	Liquid	
Monitoring of active substance concentration	Not Reported	
Method of analysis	Challenge tests / recovery tests and minimum inhibitory concentration (MIC) for bacteria and fungi.	
2.2 Reference substance		
Method of analysis for reference substance	Not applicable	
2.3 Testing procedure		

Section A5.3.1/01 Annex Point IIA, V.5.3	Efficacy Data [redacted] **MIC data and efficacy data in various products**

Test population / inoculum / test organism	*Part 1:* *Pseudomonas aeruginosa* *Naturally occurring spoilage organisms* *Part 2:* *Pseudomonas aeruginosa* *Escherichia coli* *Klebsiella pneumoniae* *Staphylococcus aureus* *Bacillus subtilis* *Proteus vulgaris* *Bacillus megaterium* *Salmonella typhosa* *Aspergillus niger* *Tricophyton mentagrophytes* *Aspergillus penicilloides* *Alternaria radicina* *Rhizopus stolonifera*
Test system	Challenge Test / Recovery Test and Minimum Inhibitory Concentration Test
Application of TS	See Appendices 1 and 2.
Test conditions	Part 1: **Challenge Test**: This test was designed to ascertain the concentration of preservative necessary to prevent microbial growth in a sample when deliberately inoculated with spoilage micro-organism. The product in each of these containers is challenged at least three times with a mixed inoculum of common spoilage organisms, in which the bacteria population is at least 1.0 x 10^6 CFU/ml. CFU= Colony Forming Units. Recovery Test: This test was designed to ascertain the concentration of preservative necessary to eliminate established microbiological contamination in spoiled samples. Inoculation: Aliquots of the product are placed in individual containers / pre-sterilized containers. Biocide (preservative) is added to the containers in such a manner as to create a ladder of samples with increasing biocide concentration. Part 2: **Minimum Inhibitory Concentration – Bacteria:** Preparation of bacterial suspension and biocide stock solution: A bacterial suspension of the organism to be prepared in an appropriate nutrient broth. A biocide stock solution is prepared by dilution of commercial product in such a manner that the concentration of the diluted solution is 20 times the

concentration of the highest biocide level to be tested. From this stock solution a series of 50% serial dilutions are made. These solutions are prepared in such a manner that upon final dilution with the bacterial suspension the biocide concentration range will encompass the desired range of the test.

Preparation of Wells:
Using a micro test well plate (96 wells), and an eight channel micropipette, a series of solutions is prepared. The first row of wells receives 190 microliters of bacterial suspension. Subsequent rows receive 100 microliters of suspension.

The first row then receives 10 microliters of the appropriate diluted biocide solution. Using the micropipette, the biocide and the bacterial suspension are mixed thoroughly.

After mixing, 100 microliters were withdrawn from the wells in the first row and transferred into the wells in the second row. The micropipette is again employed to mix the two solutions. This process is repeated until desired biocide levels have been prepared.

The well plate is then covered with its lid. The plates may be placed in a plastic bag to retard dehydration. The well plates are then incubated for 48 hours at 30ºC.

Minimum Inhibitory Concentration – Fungi:
Preparation of fungi suspension and biocide stock solution:
Grow the test fungi initially on a solid agar surface. Incubate the agar until the fungi has developed considerable growth. Flood the agar surface with sterile water and harvest the fungi by scraping it off the agar with a sterile loop. Aseptically remove the solution and transfer it into a sterile container. After transfer it may be necessary to mix the fungal suspension with shearing in order to break up the fungal mat into small pieces. Fungi which not be harvested in the manner described above may be grown in broth, either with or without aeration.

A biocide stock solution is prepared by dilution of commercial product in such a manner that the concentration of the diluted solution is 20 times the concentration of the highest biocide level to be tested. From this stock solution a series of 50% serial dilutions are made. These solutions are prepared in such a manner that upon final dilution with the bacterial suspension the biocide concentration range will encompass the desired range of the test.

Preparation of Tubes:
A sufficient number of the sterile test tubes are prepared to accommodate the desired number of biocide levels. The first row of tubes receives 8.5 millilitres of clean broth. Subsequent rows receive 4.5 millilitres of clean broth. The first row then receives 0.5 millilitres of the appropriate diluted biocide solution. The biocide and the broth are then mixed thoroughly. Following biocide addition, the first row of tubes receives 1.0 millilitres of the fungal suspension. Subsequent rows receive 0.5 millilitres of the fungal suspension.

	After mixing, 5 millilitres were withdrawn from the tubes in the first row and transferred into the tubes in the second row. The transfer pipette is employed to mix the two solutions. This process is repeated until all desired biocide levels have been prepared. The tubes are incubated at 30ºC for approximately one week.
Duration of the test / Exposure time	Part 1: Samples were challenged with standardised quantities of micro-organisms at day 1 to 7. Part 2: Minimum Inhibitory Concentration – Bacteria: 48 hours. Minimum Inhibitory Concentration – Fungi: Approximately one week.
Number of replicates performed	Part 1: One. Part 2: The entire test is normally run in duplicate. Reported results reflect the higher of the two MIC values obtained.
Controls	Part 1: Unpreserved sample of various industrial products as well as bacteria and fungi suspensions. Part 2: 0 ppm [redacted] in various bacteria and fungi suspensions.

2.4 Examination

Effect investigated	Part 1: To determine the effectiveness of [redacted] as a preservative for use in various products. Part 2: To determine the miniumum concentration of [redacted] required to inhibit the growth of specific organisms in culture media. Part 1: **Challenge Test**: Evaluation: After each challenge the efficacy of the added preservative is monitored by periodically streaking out the samples onto sterile agar plates. All plates are incubated for 48 hours at 30ºC prior to assessment. Recovery Test: The microbiology status of each sample is determined periodically after the addition of biocide by streak plating or dilution plate counting of the samples. All plates are incubated for 48 hours at 30ºC prior to assessment. The lowest level of biocide which eliminates all viable growth after the final challenge is considered to be the minimum effective concentration.

Method for recording / scoring of the effect	Part 2: **Minimum Inhibitory Concentration – Bacteria and Fungi** Readings should be reviewed to determine the well or tube with the concentration of biocide in which no growth is observed. The biocide concentration in this well or tube, is considered to be the minimum inhibitory concentration. Part 1: Streak Plates: Streak plates may be ranked in one of two ways. In the first method the plates are ranked from 0 to 5 according to the degree of microbial contamination. 0 = no growth. Not contaminated 1 = 1-5 CFU. Very lightly contaminated 2 = 6-15 CFU. Lightly contaminated 3 = 16-30 CFU. Moderately contaminated 4 = 31-45 CFU Heavily contaminated 5 = greater than 45 CFU Severely contaminated Alternatively, the plates may be ranked as either no growth, light, medium or heavy growth. - = no growth No visible colonies + = light growth A few colonies visible ++ = moderate growth Discrete colonies visible, possibly some coalescence +++= heavy/confluent growth Coalescing colonies visible throughout the streak If desired dilution plate counting may also be performed. A portion of the sample is withdrawn from each container and serially diluted until individual bacterial colonies can be counted. Each concentration is plated on sterile agar plates and incubated for 48 hours at 30ºC prior to bacterial counting. Part 2: **Minimum Inhibitory Concentration – Bacteria**: Recording test results: Plates should be placed on a mirrored plate reader and growth observed. Wells with haziness, or pellets of agglomerated material, are recorded as having growth (+). Wells with no haziness or pellets, are recorded as having no growth (-). **Minimum Inhibitory Concentration – Fungi** Recording test results: After an appropriate incubation time the tubes are examined for evidence of new growth (the original inoculation will still be visible). Tubes with new growth are recorded as having growth (+). Tubes with no new growth are recorded as having no growth (-).
Intervals of examination	Part 1: Surviving organisms were enumerated at day 1 to day 7. Part 2:

	Bacteria: Once after 48 hours. Fungi: Once after approximately one week.
Statistics	Not applicable
Post monitoring of the test organism	See data tables in Appendix 1 and 2.
	3 RESULTS
3.1 Efficacy	Part 1: Assessment of anti-microbial efficacy of [redacted] in a variety of industrial matrices. Part 2: Determination of the minimum concentration of [redacted] required to inhibit the growth of specific organisms in culture media.
Dose/Efficacy curve	Part 1: Product test concentrations ranged from 0.025 to 0.3% [redacted] Part 2: Test concentrations ranged from 6.3 to 600 ppm [redacted]
Begin and duration of effects	See Tables in Appendix 1 and 2
Observed effects in the post monitoring phase	See Tables in Appendix 1 and 2
3.2 Effects against organisms or objects to be protected	Not applicable
3.3 Other effects	Not applicable
3.4 Efficacy of the reference substance	Not applicable
3.5 Tabular and/or graphical presentation of the summarised results	Part 1: See Appendix 1, Tables on Preservative Efficacy – Viable bacteria counts Part 2: See Appendix 2, Tables on Preservative Efficacy – Minimum Inhibitory Concentrations

Metal working fluid (sample 052394)	*Pseudomonas aeruginosa*	Challenge Test	Concentration: Unpreserved, 0.05, 0.1, 0.2, 0.25 and 0.3% (% [redacted]	Result: Efficacy at 0.05% Challenge Test No visible colonies observed in any treated sample 3 days after the fourth consecutive inoculation.
Metal working fluid (sample 100694)	*Naturally occurring spoilage organisms*	Recovery Test	Concentration: Unpreserved, 0.025, 0.05, 0.075, 0.10, 0.15 and 0.2 (% [redacted]	Result: Efficacy at 0.025% Recovery Test After 2 days there no visible colonies in any of the treated samples.

NA - Bacterial /Fungal Suspension with [redacted] only.	*Pseudomonas aeruginosa* *Escherichia coli* *Klebsiella pneumoniae* *Staphylococcus aureus* *Bacillus subtilis* *Proteus vulgaris* *Bacillus megaterium* *Salmonella typhosa* *Aspergillus niger* *Tricophyton mentagrophytes* *Aspergillus penicilloides* *Alternaria radicina* *Rhizopus stolonifer*	Minimum Inhibitory Concentration (MIC) Test	600 to 6.3 ppm [redacted] and control .	MIC of [redacted] against the selected bacteria: *Pseudomonas aeruginosa: 250.0 ppm* *Escherichia coli: 31.3 ppm* *Klebsiella pneumoniae: 31.3 ppm* *Staphylococcus aureus: 18.8 ppm* *Bacillus subtilis: 7.8 ppm* *Proteus vulgaris: 9.4 ppm* *Bacillus megaterium: 18.8 ppm* *Salmonella typhosa: 18.8 ppm* MIC of [redacted] against the selected Fungi*:* *Aspergillus niger: 200 ppm* *Tricophyton mentagrophytes: 25.0 ppm* *Aspergillus penicilloides: 600.0 ppm* *Alternaria radicina: 150 ppm* *Rhizopus stolonifer: 40.0 ppm*

3.6 Efficacy limiting factors

Occurrences of resistances	Not applicable
Other limiting factors	Not applicable

4 RELEVANCE OF THE RESULTS COMPARED TO FIELD CONDITIONS

4.1 Reasons for laboratory testing

Laboratory testing of the preservative efficacy of [redacted] should mirror field test results.

4.2 Intended actual scale of biocide application

Testing was conducted on various concentrations of [redacted]
The results in the summary table in section 3.5 shows the concentrations which [redacted] provides effective preservation of metal working fluids.

4.3 Relevance compared to field conditions

Application method	Part 1. [redacted] added to metal working fluids in the concentration range of 0.025 to 0.3% [redacted] Part 2: [redacted] added to various products in the concentration range of 6.3 to 600 ppm [redacted]
Test organism	The test organisms used could be encountered in real world conditions.
Observed effect	Effective micro-organism, bacterial and fungal preservation.

4.4 Relevance for read-across

Read across required. The test method predicts the efficacy in field application.

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and methods

Preservative challenge test / recovery test and raw materials.
Minimum inhibitory concentration bacterial and fungal test and raw materials.

5.2 Reliability

Reliability = 2

5.3 Assessment of efficacy, data analysis and

The efficacy of [redacted] as an industrial preservative was investigated. The first part assessed anti-microbial efficacy of [redacted] in a variety of industrial matrices. The second part determined the minimum concentration of [redacted] required to inhibit the growth of specific organisms in

interpretation	culture media. Samples were analysed at various time points Part 1: Day 1 to 7. Part 2: Minimum Inhibitory Concentration – Bacteria: 48 hours.Minimum Inhibitory Concentration – Fungi: Approximately one week.
5.4 Conclusion	[redacted] provided effective preservation of metal working fluids as shown in Section 3.5 Summary Table.
5.5 Proposed efficacy specification	Part 1: [redacted] provided effective preservation of metal working fluids in the concentration range of 0.025 to 0.3% [redacted] Part 2: [redacted] provided effective preservation to various products in the concentration range of 6.3 to 600 ppm [redacted]

Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*December 2020*
Materials and methods	*The applicant's version is adopted.*
Conclusion	*The applicant's version is adopted.*
Reliability	*2*
Acceptability	*The method and result are acceptable.*
Remarks	*Already mentioned at the beginning of the efficacy section*

Table B5.10/01-1 Test organism

Criteria	Details
Species	*Part 1:* *Pseudomonas aeruginosa* *Naturally occurring spoilage organisms* *Part 2:* *Pseudomonas aeruginosa* *Escherichia coli* *Klebsiella pneumoniae* *Staphylococcus aureus* *Bacillus subtilis* *Proteus vulgaris* *Bacillus megaterium* *Salmonella typhosa* *Aspergillus niger* *Tricophyton mentagrophytes* *Aspergillus penicilloides* *Alternaria radicina* *Rhizopus stolonifera*
Strain	See above
Source	Not Reported
Laboratory culture	Yes
Stage of life cycle and stage of stadia	Not applicable
Mixed age population	No
Other specification	Not applicable
Number of organisms tested	Part 1: Metal working fluid (sample 052394): One *Pseudomonas aeruginosa* Metal working fluid (sample 100694): One Naturally occurring spoilage organisms Part 2: Thirteen
Method of cultivation	Bacteria - incubation 48 hours at 30°C Fungi – incubation at 30°C for approximately one week
Pretreatment of test organisms before exposure	No
Initial density/number of test organisms in the test system	At least 1.0×10^6 cfu/ml

Table B5.10/01-2 Application of test substance

Criteria	Details
Application procedure	[redacted] added to various industrial products
Delivery method	Not applicable
Dosage rate	Part 1: Product test concentrations ranged from 0.025 to 0.3% [redacted] Part 2: Test concentrations ranged from 6.3 to 600 ppm [redacted]
Carrier	Not Reported
Concentration of liquid carrier	Not Reported
Liquid carrier control	Not applicable
Other procedures	Not applicable

Table B5.10/01-3 Test conditions

Criteria	Details
Substrate	Dilution of various industrial products
Incubation temperature	30°C
Moisture	Not known
Aeration	None Reported
Method of exposure	Individual dilutions of [redacted]
Aging of samples	Not Reported
Other conditions	None

Appendix 1

Test Type: Challenge
Organisms Used: Pseudomonas aeruginosa

Application: Metalworking Fluid (Sample 052394)
Result Found: Efficacy at 0.05%

SAMPLE/ TREATMENT	VIABLE BACTERIA AFTER 1ST INOC TIME IN DAYS 10^8 CFU/ML			2ND INOC TIME IN DAYS 10^8CFU/ML			3RD INOC TIME IN DAYS 10^9CFU/ML			4TH INOC TIME IN DAYS 10^9CFU/ML		
	1	3	7	1	3	7	1	3	7	1	3	7
[redacted]												
0.05%	-	-	-	-	-	-	+	+	-	+	-	-
0.1%	-	-	-	-	-	-	-	-	-	-	-	-
0.2%	-	-	-	-	-	-	-	-	-	-	-	-
0.25%	-	-	-	-	-	-	-	-	-	-	-	-
0.3%	-	-	-	-	-	-	-	-	-	-	-	-
Control	+++	-	-	+++	-	-	+++	++	+	+++	+++	+++

\- = no growth (no visible colonies)

\+ = light growth (a few colonies visible)

++ = moderate growth (discrete colonies visible, possibly some coalescence)

+++ = dense/confluent growth (coalescing colonies visible throughout streak)

Test Type: Recovery
Organisms Used: Naturally Occuring Spoilage Organisms

Application: Metalworking Fluid (Sample 100694)
Result Found: Efficacy at 0.025%

RECOVERY TEST				
Concentration of [redacted] (%)	Aerobic Bacterial Counts Streak Plate and Dilution Plate Count Results Days Post Inoculation			
	1	2	3	7
control	5	5	$1.5x10^6$	$1.8x10^6$
0.025	2	0	<10	<10
0.05	1	0	<10	<10
0.075	0	0	<10	<10
0.10	0	0	<10	<10
0.15	0	0	<10	<10
0.20	0	0	<10	<10

Streak plates are ranked from 0-5 according to the degree of microbial growth.
0 = no growth
1 = 1-5 Colony Forming Unit (CFU)
2 = 6-15 CFU
3 = 16-30 CFU
4 = 31-45 CFU
5 = greater than 45 CFU
Serial dilution counts are expressed as Colony forming Units per gram (CFU/g).

Appendix 2

Minimum Inhibitory Concentration (MIC) of [redacted] Against Selected Bacteria.

[redacted] (ppm)	Bacteria		
	Pseudomonas aeruginosa	Escherichia coli	Klebsiella pneumoniae
0	+	+	+
6.3	+	+	+
7.8	+	+	+
9.4	+	+	+
12.5	+	+	+
15.6	+	+	+
18.8	+	+	+
25.0	+	+	+
31.3	+	-	-
37.5	+	-	-
50.0	+	-	-
62.5	+	-	-
75.0	+	-	-
100.0	+	-	-
125.0	+	-	-
150.0	+	-	-
200.0	+	-	-
250.0	-	-	-
300.0	-	-	-
400.0	-	-	-
500.0	-	-	-
600.0	-	-	-

Minimum Inhibitory Concentration (MIC) of [redacted] Against Selected Bacteria.

[redacted] (ppm)	Bacteria		
	Staphylococcus aureus	Bacillus subtilis	Proteus vulgaris
0	+	+	+
6.3	+	+	+
7.8	+	-	+
9.4	+	-	-
12.5	+	-	-
15.6	+	-	-
18.8	-	-	-
25.0	-	-	-
31.3	-	-	-
37.5	-	-	-
50.0	-	-	-
62.5	-	-	-
75.0	-	-	-
100.0	-	-	-
125.0	-	-	-
150.0	-	-	-
200.0	-	-	-
250.0	-	-	-
300.0	-	-	-
400.0	-	-	-
500.0	-	-	-
600.0	-	-	-

Minimum Inhibitory Concentration (MIC) of [redacted] Against Selected Bacteria.

[redacted] (ppm)	Bacteria		
	Bacillus megaterium	Salmonella typhosa	
0	+	+	
6.3	+	+	
7.8	+	+	
9.4	+	+	
12.5	+	+	
15.6	+	+	
18.8	-	-	
25.0	-	-	
31.3	-	-	
37.5	-	-	
50.0	-	-	
62.5	-	-	
75.0	-	-	
100.0	-	-	
125.0	-	-	
150.0	-	-	
200.0	-	-	
250.0	-	-	
300.0	-	-	
400.0	-	-	
500.0	-	-	
600.0	-	-	

Minimum Inhibitory Concentration (MIC) of [redacted] Against Selected Fungi.

[redacted] (ppm)	Fungi		
	Aspergillus niger	Tricophyton mentagrophytes	Aspergillus penicilloides
0	+	+	+
6.3	+	+	+
7.8	+	+	+
9.4	+	+	+
12.5	+	+	+
15.6	+	+	+
18.8	+	+	+
25.0	+	-	+
31.3	+	-	+
37.5	+	-	+
50.0	+	-	+
62.5	+	-	+
75.0	+	-	+
100.0	+	-	+
125.0	+	-	+
150.0	+	-	+
200.0	-	-	+
250.0	-	-	+
300.0	-	-	+
400.0	-	-	+
500.0	-	-	+
600.0	-	-	-

Minimum Inhibitory Concentration (MIC) of [redacted] Against Selected Fungi.

[redacted] (ppm)	Alternaria radicina		Proxel BD20 (ppm)	Rhizopus stolonifer
0	+		0	+
9.4	+		20.0	+
18.8	+		40.0	-
37.5	+		79.0	-
75.0	+		157.0	-
150.0	-		313.0	-
300.0	-		625.0	-
600.0	-		1250.0	-

Section A5.3.1/02 Annex Point IIA, V.5.3 **Efficacy Data**

[redacted] efficacy testing and performance in various products: Supplemental Data

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1 REFERENCE

1.1 Reference	[redacted] efficacy testing and performance in various products: Supplemental Data, [redacted] 1992. [redacted] efficacy and performance in various products, [redacted] 1991.
1.2 Data protection	Yes
Data owner	Lonza Cologne GmbH
Companies with letter of access	
Criteria for data protection	Data on existing substance for first entry into Annex I.
1.3 Guideline study	None.
1.4 Deviations	None stated.

2 METHOD

2.1 Test Substance (Biocidal Product)	[redacted]
Trade name/ proposed trade name	
Composition of Product tested	1,2-benzisothiazol-3(2H)-one at 20 % active ingredient
Physical state and nature	Liquid
Monitoring of active substance concentration	Not Reported
Method of analysis	**Recovery Tests:** This report contains efficacy data on [redacted] as an industrial

preservative. This data is supplemental to efficacy data supplied in "[redacted] Efficacy Testing and Performance in Various Products", September 20, 1991 by [redacted]

Recovery testing referred to herein were previously described.

Minimum Inhibitory Concentration Test (MIC) Tests:
All cultures were obtained from the American Type Culture Collection. Bacterial inocula were grown in tryptic soy broth at 30ºC. Fungal inocula were grown in Sabourand dextrose broth at ambient temperature. A ladder of concentrations of the respective biocides was made in the same medium used for growth and inoculated with an actively growing culture. The reported MIC is the lowest concentration that yielded no visible growth at 48 hours.

2.2 Reference substance

Method of analysis for reference substance: Not applicable

2.3 Testing procedure

Test population / inoculum / test organism:

Challenge Test/Recovery Test:
The following bacteria are utilised for these evaluations:

Pseudomonas aeruginosa	ATCC*[redacted]
Enterobacter cloacae	ATCC*[redacted]
Escherichia coli	ATCC*[redacted]
Acinetobacter calcoaceticus	ATCC*[redacted]

*ATCC: American Type Culture collection number.

See Section 2.1.5.

MIC Test:

Current Name:	Old Name:
Bacteria:	
Bacillus megterium	same
Salmonella typhi	*Salmonella typhosa*
Klebsiella pneumoniae	*Aerobacter aerogenes*
Fungi:	
Aspergillus penicilloides	*Aspergillus glaucus*
Rhizopus stolonifer	*Rhizopus nigricans*
Alternaria radicina	same
Trichophyton mentagrophytes	same

Test system: Recovery tests in micro-organism and minimum inhibitory concentration (MIC) in bacteria and fungi.

Section A5.3.1/02 Annex Point IIA, V.5.3 **Efficacy Data**

[redacted] efficacy testing and performance in various products: Supplemental Data

Application of TS	See Appendices 1 and 2.
Test conditions	See Section 2.1.5.
Duration of the test / Exposure time	See Section 2.1.5.
Number of replicates performed	See Section 2.1.5.
Controls	See Section 2.1.5.

2.4 Examination

Effect investigated	To determine the effectiveness of [redacted] as a preservative for use in metal working fluid products.
Method for recording / scoring of the effect	See Section 2.1.5.
Intervals of examination	See Section 2.1.5.
Statistics	Not applicable
Post monitoring of the test organism	See data tables in Appendix 1 and 2.

3 RESULTS

3.1 Efficacy	**Recovery Tests**: Assessment of anti-microbial efficacy of [redacted] in an industrial matrix. **MIC Test:** Determination of the minimum concentration of [redacted] required to inhibit the growth of specific organisms in culture media.
Dose/Efficacy curve	**Recovery Tests**: Product test concentrations ranged from 250-3000 ppm [redacted] **MIC Test:** Test concentrations ranged from 10 to 300 ppm [redacted]
Begin and duration of effects	See Tables in Appendix 1 and 2
Observed effects in the post	See Tables in Appendix 1 and 2

monitoring phase	
3.2 Effects against organisms or objects to be protected	Not applicable
3.3 Other effects	Not applicable
3.4 Efficacy of the reference substance	Not applicable
3.5 Tabular and/or graphical presentation of the summarised results	See Appendix 1, Tables on Preservative Efficacy – Recovery Tests See Appendix 2, Tables on Preservative Efficacy – MIC Test

Metal Working Fluid	*Pseudomonas aeruginosa* *Enterobacter cloacae* *Escherichia coli* *Acinetobacter calcoaceticus*	Recovery Test	Concentration: Unpreserved, 250, 500, 750, 1000, 1250, 1500, 2000, 2500, and 3000 ppm [redacted]	Result: Efficacy at 1250 ppm (0.125)%

Bacterial /Fungal Suspension with [redacted] only.	*Bacteria* *Bacillus megaterium* *Salmonella tvphi* *Klebsiella pneumonia* *Fungi* *Aspergillus penicilloides* *Rhizopus stolonifer* *Alternaria radicina* *Trichophyton mentagrophytes*	Minimum Inhibitory Concentration (MIC) Test	Concentration: Unpreserved, 10, 25, 50, 100, 150, 200, 250 and 300, ppm [redacted]	MIC of [redacted] against selected bacteria, fungi and yeast Bacteria *Bacillus megaterium: 25 ppm* *Salmonella tvphi: 25 ppm* *Klebsiella pneumoniae: 50 ppm* *Fungi* *Aspergillus penicilloides: 50 ppm* *Rhizopus stolonifer: 100 ppm* *Alternaria radicina|: 50 ppm* *Trichophyton mentagrophytes: 50 ppm*

3.6 Efficacy limiting factors

Occurrences of resistances: Not applicable

Other limiting factors: Not applicable

4 RELEVANCE OF THE RESULTS COMPARED TO FIELD CONDITIONS

4.1 Reasons for laboratory testing

Laboratory testing of the preservative efficacy of [redacted] should mirror field test results.

4.2 Intended actual scale of biocide application

Testing was conducted on various concentrations of [redacted]

The results in the summary table in section 3.5 shows the concentrations which [redacted] provides effective preservation of the industrial products.

4.3 Relevance compared to field conditions

Application method

Recovery Tests:

Product test concentrations ranged from 250-3000 ppm [redacted]

MIC Test:

Test concentrations ranged from 10 to 300 ppm [redacted]

Test organism	The test organisms used could be encountered in real world conditions.
Observed effect	Effective micro-organism, bacterial and fungal preservation.
4.4 Relevance for read-across	Read across required. The test method predicts the efficacy in field application.

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and methods	Preservative recovery test and raw materials. Minimum inhibitory concentration bacterial and fungal test and raw materials.
5.2 Reliability	Reliability = 2
5.3 Assessment of efficacy, data analysis and interpretation	The efficacy of [redacted] as an industrial preservative was investigated. The first part assessed anti-microbial efficacy of [redacted] in an industrial matrix. The second part determined the minimum concentration of [redacted] required to inhibit the growth of specific organisms in culture media. Samples were analysed at various time points **Recovery Test**: Day 1, 2 and 3 and including Day 7. **Minimum Inhibitory Concentration** – Bacteria: 48 hours. **Minimum Inhibitory Concentration** – Fungi: 48 hours.
5.4 Conclusion	[redacted] provided effective preservation of an industrial product is shown in Section 3.5 Summary Table. Considering the worst case, the study demonstrates bactericidal activity at 50 ppm of the biocidal product acts against *Klebsiella pneumoniae*, fungicidal activity at 100 ppm of the biocidal product acts against *Rhizopus stolonifera*.
5.5 Proposed efficacy specification	**Recovery Tests**: [redacted] provided effective preservation to the product in the concentration range of 1250 to 3000 ppm [redacted] **MIC Test**: [redacted] provided effective preservation to the product in the concentration range of 50 to 300 ppm [redacted] for bacteria and 100 to 300 ppm [redacted] for fungi.

Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*December 2020*
Materials and methods	*The applicant's version is adopted.*
Conclusion	*The applicant's version is adopted.*
Reliability	*2*
Acceptability	*The method and result are acceptable.*
Remarks	*Already mentioned at the beginning of the efficacy section*

Table B5.10/01-1 Test organism

Criteria	Details
Species	**Challenge/Recovery Tests:** *Pseudomonas aeruginosa* ATCC* [redacted] *Enterobacter cloacae* ATCC* [redacted] *Escherichia coli* ATCC* [redacted] *Acinetobacter calcoaceticus* ATCC* [redacted] *ATCC: American Type Culture collection number. **MIC Test:** Bacteria *Bacillus megaterium* *Salmonella tvphi* *Klebsiella pneumoniae* Fungi *Aspergillus penicilloides* *Rhizopus stolonifer* *Alternaria radicina* *Trichophyton mentagrophytes*
Strain	See above
Source	American Type Culture Collection
Laboratory culture	Yes
Stage of life cycle and stage of stadia	Not applicable
Mixed age population	No
Other specification	Not applicable
Number of organisms tested	**Challenge/Recovery Tests:** Four **MIC Test:** Seven
Method of cultivation	Incubation 48 hours at 30°C
Pretreatment of test organisms before exposure	No
Initial density/number of test organisms in the test system	At least 1.0 x 10^2 cfu/g

Table B5.10/01-2 Application of test substance

Criteria	Details

Application procedure	[redacted] added to various industrial products
Delivery method	Not applicable
Dosage rate	**Challenge/Recovery Tests**: Product test concentrations ranged from 250-3000 ppm [redacted] **MIC Test:** Test concentrations ranged from 10 to 300 ppm [redacted]
Carrier	Not Reported
Concentration of liquid carrier	Not Reported
Liquid carrier control	Not applicable
Other procedures	Not applicable

Table B5.10/01-3 Test conditions

Criteria	**Details**
Substrate	Dilution of various industrial products
Incubation temperature	30°C
Moisture	Not known
Aeration	None Reported
Method of exposure	Individual dilutions of [redacted]
Aging of samples	See Section 2.1.5
Other conditions	See Section 2.1.5

Appendix 1

Efficacy of [redacted] as a Preservative for a Metal Working Fluid

ppm BIOCIDE [redacted]	Viable Microorganisms / gram DAYS POST BIOCIDE ADDITION 1	2	3	7
0	5	5	3.74×10^7	5
250	5	5	2.47×10^7	5
500	5	5	1.99×10^7	5
750	5	5	1.93×10^7	5
1000	5	4	2.9×10^4	5
1250	5	3	1.5×10^2	0
1500	3	0	< 10	1
2000	1	0	< 10	1
2500	1	1	< 10	1
3000*	1	0	< 10	0

Appendix 2

Minimum Inhibitory Concentration (MIC) of [redacted] against selected bacteria.

	bacteria		
ppm [redacted]	Bacillus megaterium	Salmonella typhi	Klebsiella pneumonia
0	+	+	+
10	+	+	+
25	-	-	+
50	-	-	-
100	-	-	-
150	-	-	-
200	-	-	-
250	-	-	-
300	-	-	-

Minimum Inhibitory Concentration (MIC) of [redacted] against selected fungi.

	fungi			
ppm [redacted]	Aspergillus penicilloides	Rhizopus stolonifer	Alternaria radicina	Trichophyton mentagrophytes
0	+	+	+	+
10	+	+	+	+
25	+	+	+	+
50	-	+	-	-
100	-	-	-	-
150	-	-	-	-
200	-	-	n.d.	-
250	-	-	n.d.	-
300	-	-	n.d.	-

Official use only

1 REFERENCE

1.1	**Reference**	[redacted] MWF Efficacy; [redacted] Metal Working Fluid Recovery Tests, [redacted] 1994, Not GLP, Unpublished.
1.2	**Data protection**	Yes
Data owner		Lonza Cologne GmbH
Companies with letter of access		
Criteria for data protection		Data on existing substance for first entry into Annex I.
1.3	**Guideline study**	None.
1.4	**Deviations**	None stated.

2 METHOD

2.1	**Test Substance (Biocidal Product)**	[redacted]
Trade name/ proposed trade name		
Composition of Product tested		1,2-benzisothiazol-3(2H)-one at 20 % active ingredient
Physical state and nature		Liquid
Monitoring of active substance concentration		Not Reported
Method of analysis		Recovery tests
2.2	**Reference substance**	
Method of analysis for reference substance		Not applicable
2.3	**Testing procedure**	
Test population / inoculum / test organism		Natural isolates from spoiled product See Table in section 3.5.

Section A5.3.1/03 Annex Point IIA, V.5.3	Efficacy Data [redacted] Metal Working Fluid Recovery Tests

Test system	Recovery Test
Application of TS	See Appendices 1, 2 and 3.
Test conditions	Part 1: G165 central system sample was received contaminated. Approximately 3.4 x 10^5 bacterial organisms were detected. 13 ppm [redacted] was also recovered from this product on receipt. This concentration represents a significant difference from the 80 ppm that had been indicated was the dosage level. Investigation to ensure adequate dosing may be required on this information. Part 2: Microbiological evaluations on the microgrind fluid from Rexnord. This sample was received initially contaminated containing 1.56 x 10^3 viable bacteri/mL. Approximately 10 % of an inoculum was added of 4 standard laboratory organisms to increase growth in the sample and allow proper evaluation. Part 3: Recovery testing with the steel rolling emulsion sent to the laboratory was completed [redacted] The sample was received containing 1.2 x 10^5 bacteria per gram. These organisms were determined to be of the non-spore-forming type. Several fungal colonies were also detected in this sample.
Duration of the test / Exposure time	Part 1: 1, 3 and 7 Days post biocide addition Part 2: 1, 2, 3, 7 and 9 Days post biocide addition Part 3: 1, 2, 3, 7 Days post biocide addition
Number of replicates performed	Part 1: See Appendix 1 Part 2: See Appendix 2 Part 3: See Appendix 3
Controls	Part 1: Unpreserved samples, see Appendix 1 Part 2: Unpreserved samples, see Appendix 2 Part 3: Unpreserved samples, see Appendix 3

2.4 Examination

Effect investigated	To determine the effectiveness of [redacted] as a preservative for use in various products. Part 1: See section 3.5 and Appendix 1. The efficacy status of each sample (Metal Working Fluid Sample G165) is determined periodically after the addition of biocide ([redacted] by assessing the number of micro-organsisms present in each sample. Part 2: See section 3.5 and Appendix 2. The efficacy status of each sample (Metal Working Fluid-Microgrind 573-PTC) is determined periodically after the addition of biocide ([redacted] by

assessing the number of micro-organsisms present in each sample.

Part 3:
Initial Microbiological Status
On receipt, the samples were checked for the presence of viable micro-organisms using a serial dilution plate counting technique on Tryptone Glucose Extract Agar (TGEA). After incubation for 48 hours at 30ºC, the following results were generated regarding microbial contamination.

Sample	Viable micro-organisms per gram
B1003-93	1.2×10^5*

*fungal colonies were also detected in this sample.

Heat Shock Test
Bacteria detected in samples were checked for their ability to produce spores by raising the temperature of a broth containing these organisms to 85ºC for fifteen minutes.

The ability of an organism to withstand such a heat shock is indicative of those organisms capable of producing preservative resistant spores.

Sample	Initial	Post 15 mins. @ 85ºC	Spores
ATCC 11229†	1.41×10^9	< 10	No
ATCC 27328‡	9.1×10^8	1.22×10^8	Yes

† non-spore-forming *Escherichia coli* to serve as a NEGATIVE control
‡ spore-forming *Bacillus subtilis* to serve as a POSITIVE control

Recovery Test
This test is designed to ascertain the concentration of preservative necessary to eliminate established microbiological contamination in your samples.

Preparation of the Preservation Series
Forty grams of sample were dispensed into pre-sterilized containers. A series of concentrations of [redacted] were then added to these samples. Once sample was left untreated to act as a preservative-free control.

Inoculation and Evaluations
The microbiological status of each sample was determined 1, 2, 3 and 7 days after [redacted] addition by "streaking out" or dilution plate counting the sample on plates of TGEA.

The results of this test are illustrated in Appendix 3.

Method for recording / scoring of the effect

Part 1, Part 2 and Part 3:
Recovery Test

0 = no growth.
1 = 1-5 CFU (Colony Forming Units)
2 = 6-15 CFU
3 = 16-30 CFU
4 = 31-45 CFU
5 = greater than 45 CFU

	<10 = No detectable bacterial colonies Dilution counts (cfu/g)
Intervals of examination	Part 1: 1, 3 and 7 Days post biocide addition Part 2: 1, 2, 3, 7 and 9 Days post biocide addition Part 3: 1, 2, 3, 7 Days post biocide addition
Statistics	Not applicable
Post monitoring of the test organism	See data tables in Appendix 1, 2 and 3.

3 RESULTS

3.1 Efficacy

Part 1:

Recovery testing using [redacted] in this fluid was sucessful. Table in Appendix 1 reports the data from the evaluations. It was determined that [redacted] was effective in eliminating contamination at 500 ppm (0.05 % w/w) following 72 hours after addition.

The concentration of [redacted] which is effective in recovering a spoiled system usually represents a 100-200% increase over the amount of [redacted] needed to preserve a relatively clean system. Therefore, although it is generally recommended a minimum of 500 ppm to protect most industrial systems, 150-250 ppm [redacted] may be adequate in keeping the fluid preserved, especially since it was indicated that this system is a daily "once through" application and not a recirculating one.

Part 2:

Recovery testing with this product revealed that 1250 ppm [redacted] was effective in eliminating all organisms following 24 hours after biocide addition in Table in Appendix 2. This test simulates a scenario in which grossly contaminated fluid will be recovered. In a situation where protection of an essentially clean fluid is required, lower dosages are usually effective based on the fact that the organisms do not have sufficient time to become apapted to the product.

Part 3:

Recovery testing with this product determined that [redacted] will effectively eliminate the organisms detected in this system after 7 days at a concentration of 500 parts per million (0.05 % w/w of product). All testing was conducted at 110ºF as requested.

It was determined that a higher concentration of [redacted] 1250 ppm, will also effect a complete kill within 72 hours of addition see table in Appendix 3. It was recommeded that a dosage of [redacted] in this range be used, depending on the effect time desired, to restore this product and prevent further contamination.

Dose/Efficacy curve

Part 1:
Product test concentrations ranged from 0, 50, 80, 100, 250, 500, 1000 and 2000 ppm [redacted]
Part 2:
Product test concentrations ranged from 0, 500, 750, 1000, 1250, 1500, 2000 and 2500 ppm [redacted]

		Part 3: Product test concentrations ranged from 0, 100, 250, 500, 750, 1000, 1250, 1500, 2000 and 2500 ppm [redacted]
Begin and duration of effects		See Tables in Appendix 1, 2 and 3
Observed effects in the post monitoring phase		See Tables in Appendix 1, 2 and 3
3.2	**Effects against organisms or objects to be protected**	Not applicable
3.3	**Other effects**	Not applicable
3.4	**Efficacy of the reference substance**	Not applicable
3.5	**Tabular and/or graphical presentation of the summarised results**	Part 1: See Appendix 1, Table on Preservative Efficacy Part 2: See Appendix 2, Table on Preservative Efficacy Part 3: See Appendix 3, Table on Preservative Efficacy

Field of Use Envisaged	Test Organism(s)	Test Method	Test Conditions	Test Results: Effects, Mode of action, resistance
Metal Working Fluid Sample G165	Natural isolate from spoiled product	Recovery Test (7 days Post Inoculation)	Concentration: Unpreserved, 50, 80, 100, 250, 500, 1000 and 2000 ppm	Result: Efficacy at 500 ppm (0.05%)

Metal Working Fluid-Microgrind 573-PTC	Natural isolate from spoiled product (1.56×10^3 viable bacteria/ml) and 10% Standard Laboratory Inoculum	Recovery Test (9 Days Post Inoculation)	Concentration: Unpreserved, 500, 750, 1000, 1250, 1500, 2000 and 2500 ppm	Result: Efficacy at 1250 ppm (0.125%)
Steel Rolling Emulsion (Sample B1003-93)	Natural isolate from spoiled product (1.2×10^5 viable bacteria/g and fungal colonies)	Recovery Test (7 Days Post Inoculation) at 110 °F (43.3 °C)	Concentration: Unpreserved, 100, 250, 500, 750, 1000, 1250, 1500, 2000 and 2500 ppm ([redacted]	Result: Efficacy at 500 ppm (0.05%)

3.6 Efficacy limiting factors

Occurrences of resistances: Not applicable

Other limiting factors: Not applicable

4 RELEVANCE OF THE RESULTS COMPARED TO FIELD CONDITIONS

4.1 Reasons for laboratory testing

Laboratory testing of the preservative efficacy of [redacted] should mirror field test results.

4.2 Intended actual scale of biocide application

Testing was conducted on various concentrations of [redacted]

The results in the summary table in section 3.5 shows the concentrations which [redacted] provides effective preservation of the various products.

4.3 Relevance compared to field conditions

Application method

Part 1.

[redacted] added to products in the concentration range of 0 to 2000 ppm [redacted]

Part 2:

[redacted] added to products in the concentration range of 0 to 2500 ppm [redacted]

Part 3:

[redacted] added to products in the concentration range of 0 to 2500 ppm [redacted]

Section A5.3.1/03 Annex Point IIA, V.5.3	**Efficacy Data** [redacted] **Metal Working Fluid Recovery Tests**

Test organism		The test organisms used could be encountered in real world conditions.
Observed effect		Effective micro-organism preservation.
4.4	**Relevance for read-across**	Read across required. The test method predicts the efficacy in field application.
	5 APPLICANT'S SUMMARY AND CONCLUSION	
5.1	**Materials and methods**	Preservative recovery test and raw materials.
5.2	**Reliability**	Reliability = 2
5.3	**Assessment of efficacy, data analysis and interpretation**	The efficacy of [redacted] as a preservative was investigated. Samples were analysed at various time points Part 1: Day 1 to 7. Part 2: Day 1 to 9. Part 3: Day 1 to 7.
5.4	**Conclusion**	[redacted] provided effective preservation of a various products as shown in Section 3.5 Summary Table.
5.5	**Proposed efficacy specification**	Part 1: [redacted] provided effective preservation to a Metal Working Fluid Sample G165 at the concentration of at 500 ppm (0.05%) ppm [redacted] Part 2: [redacted] provided effective preservation to a Metal Working Fluid-Microgrind 573-PTC at the concentration of 1250 ppm (0.125%) ppm [redacted] Part 3: [redacted] provided effective preservation to a Steel Rolling Emulsion (Sample B1003-93) at the concentration of 500 ppm (0.05%) ppm [redacted]

Evaluation by Competent Authorities	
EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*December 2020*
Materials and methods	*The applicant's version is adopted.*
Conclusion	*The applicant's version is adopted.*
Reliability	*2*
Acceptability	*The method and result are acceptable.*

Section A5.3.1/03 Annex Point IIA, V.5.3	**Efficacy Data** [redacted] **Metal Working Fluid Recovery Tests**

Remarks	*Already mentioned at the beginning of the efficacy section*

Table B5.10/01-1 Test organism

Criteria	Details
Species	*Natural isolate from spoiled product*
Strain	See above
Source	Not Reported
Laboratory culture	Yes
Stage of life cycle and stage of stadia	Not applicable
Mixed age population	No
Other specification	Not applicable
Number of organisms tested	See Table in Section 3.5 and Appendices 1, 2 and 3.
Method of cultivation	Part 3 - incubation 48 hours at 30°C
Pretreatment of test organisms before exposure	No
Initial density/number of test organisms in the test system	See Section 2.3.4

Table B5.10/01-2 Application of test substance

Criteria	Details
Application procedure	[redacted] added to various industrial products
Delivery method	Not applicable
Dosage rate	Part 1: Product test concentrations ranged from 0, 50, 80, 100, 250, 500, 1000 and 2000 ppm [redacted] Part 2: Product test concentrations ranged from 0, 500, 750, 1000, 1250, 1500, 2000 and 2500 ppm [redacted] Part 3: Product test concentrations ranged from 0, 100, 250, 500, 750, 1000, 1250, 1500, 2000 and 2500 ppm [redacted]
Carrier	Not applicable

Concentration of liquid carrier	Not applicable
Liquid carrier control	Not applicable
Other procedures	Not applicable

Table B5.10/01-3 Test conditions

Criteria	**Details**
Substrate	Dilution of various products
Incubation temperature	30°C
Moisture	Not known
Aeration	None Reported
Method of exposure	Individual dilutions of [redacted]
Aging of samples	Not Reported
Other conditions	None

Appendix 1

TABLE 1

RECOVERY TEST RESULTS

[redacted]

Viable Microorganisms
gram

ppm BIOCIDE [redacted]	DAYS POST BIOCIDE ADDITION 1	3	7
0 (control)	5	5	4.4×10^6
50	5	5	1.24×10^7
80	5	5	5.0×10^6
100	5	5	4.9×10^6
250	5	5	3.2×10^6
500	5	0	< 10
1000	2	0	< 10
2000	1	0	< 10

0 = No Growth
1 = 1-5 CFU (Colony Forming Units)
2 = 6-15 CFU
3 = 16-30 CFU
4 = 31-45 CFU
5 = Greater than 45 CFU

< 10 = No detectable bacterial colonies

Appendix 2

TABLE 1

RECOVERY TEST RESULTS

[redacted]

Viable Microorganisms
gram

ppm BIOCIDE [redacted]	DAYS POST BIOCIDE ADDITION 1	2	3	7	9
0	5	5	$8.6x10^5$	$6.8x10^4$	$3.5x10^4$
500	1	2	< 10	$1.37x10^5$	$4.8x10^4$
750	1	0	< 10	6.2×10^4	3.8×10^4
1000	0	1	< 10	$5.7x10^2$	$8.6x10^2$
1250	0	0	< 10	< 10	< 10
1500	0	0	< 10	< 10	< 10
2000	0	0	< 10	< 10	< 10
2500	0	0	< 10	< 10	< 10

0 = No Growth
1 = 1-5 CFU (Colony Forming Units)
2 = 6-15 CFU
3 = 16-30 CFU
4 = 31-45 CFU
5 = Greater than 45 CFU

< 10 = No detectable bacterial colonies

Section A6 Toxicological and Metabolic Studies

Subsection A6.1.1/1 Acute Toxicity

Annex Point IIA VI.6.1.1 6.1.1 Acute oral toxicity in rats (LD_{50} test)

			Official use only
		1 REFERENCE	
1.1	**Reference**	[redacted] 1988; [redacted] acute oral toxicity to the rat. [redacted] Report No. [redacted]P/2079; GLP; Unpublished	
1.2	**Data protection**	Yes	
1.1	Data owner	Arch Chemicals Inc	
1.2	Companies with letter of access	Clariant Production UK Ltd, Thor GmbH	
1.3	Criteria for data protection	Data on existing substance for first entry into Annex I.	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	Not stated Method used comparable to guidelines OECD 401, EC B.1	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	Reference [redacted]	
3.1.2	Specification	As given in section 2	
3.1.2.1	Purity	73.1%	
3.1.2.2	Stability	Not applicable (single administration)	
3.2	**Test Animals**		
3.2.1	Species	Rat	
3.2.2	Strain	Wistar-derived albino (Alpk:APfSD)	
3.2.3	Source	[redacted]	

Section A6 Toxicological and Metabolic Studies

Subsection A6.1.1/1 Acute Toxicity

Annex Point IIA VI.6.1.1 6.1.1 Acute oral toxicity in rats (LD_{50} test)

3.2.4	Sex	♂♀	
3.2.5	Age/weight at study initiation	♂	240-267 g
		♀	182-206 g
3.2.6	Number of animals per group	♂	5
		♀	5
3.2.7	Control animals	No	
3.3	**Administration/ Exposure**	Oral	
3.3.1	Postexposure period	14 days	
3.3.2	Type	Gavage	
3.3.3	Doses	100, 300, 500 and 900 mg/kg bw	
3.3.4	Vehicle	0.5% w/v aqueous polysorbate 80	
3.3.5	Concentration in vehicle	10, 30, 50, 90 mg/mL nominal, 9.5, 28.8, 47.4, 92.9 mg/mL measured	
3.3.6	Total volume applied	10 mL/kg. The dose-volume was calculated for each animal according to its weight at the time of dosing.	
3.3.7	Control	No controls included in the experimental design	
3.4	**Examinations**	The animals were weighed on the day before dosing, the day of dosing (Day 1) and on Day 3, Day 4, Day 8 and Day 15. In addition, surviving females from the top-dose group were weighed on Days 2 and 6 (but not on Day 4). The animals were observed for signs of systemic toxicity once between 30 and 60 minutes after dosing and twice between 2.5 and 5 hours after dosing. Subsequent observations were made once daily up to Day 15 (surviving top-dose females were not observed on Day 11). Animals in extremis and those surviving at the end of the study were humanely killed by inhalation of excessive levels of halothane BP vapour followed by cervical dislocation and were examined by necropsy for any macroscopic abnormalities.	

Section A6 Toxicological and Metabolic Studies

Subsection A6.1.1/1 Acute Toxicity

Annex Point IIA VI.6.1.1 6.1.1 Acute oral toxicity in rats (LD_{50} test)

3.5	**Method of determination of LD_{50}**	♂ Linear log dose interpolation ♀ Probit method (Finney, 1971)
3.6	**Further remarks**	Results based on Nominal dose values, not adjusted for purity.

4 RESULTS AND DISCUSSION

4.1	**Mortality**	There were no deaths in animals dosed with 100 or 300 mg/kg. One female dosed with 500 mg/kg was killed *in extremis* on Day 2. All the males and three females dosed with 900 mg/kg died or were killed *in extremis* on Days 1 or 2. There were no signs of toxicity at any time in the animals dosed with 100 mg/kg. Signs of slight toxicity in those dosed with 300 mg/kg were piloerection and upward curvature of the spine, neither of which persisted after Day 3. Surviving animals dosed with 500 mg/kg also showed signs of slight toxicity until Day 3. These were of a slightly higher incidence than those seen following dosing with 300 mg/kg, and included dehydration, piloerection and upward curvature of the spine. Signs of marked toxicity were observed in the animals dosed with 900mg/kg. The most common abnormalities were upward curvature of the spine, piloerection, sides pinched-in, dehydration, hypothermia and decreased activity. Two animals appeared cyanosed prior to death. The two surviving females had recovered by days 6 or 7.
4.2	**Clinical signs**	There were no macroscopic abnormalities in any animal at necropsy.
4.3	**Pathology**	All animals showed an initial bodyweight loss, due to the pre-dose fast. All of those dosed with 100 mg/kg, and most of those dosed with 300 mg/kg, had started to gain weight by Day 3. Most surviving animals dosed with 500 mg/kg had gained weight by Day 4, and the two surviving females dosed with 900 mg/kg had gained weight by Day 8. The overall weight gain, throughout the study, was similar at all dose-levels.
4.4	**Other**	♂ 670 mg/kg (approximate 95% confidence limits 500, 900) ♀ 784 mg/kg (lower 95% confidence limit 475) Adjusted for 73.1% purity ♂ 490 mg/kg ♀ 573 mg/kg

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1	**Materials and methods**	Groups of five male and five female rats were given a single oral dose of 100, 300, 500 or 900 mg/kg of [redacted] as preparations in 5% (w/v) aqueous polysorbate 80. The animals were

Section A6 Toxicological and Metabolic Studies

Subsection A6.1.1/1 Acute Toxicity

Annex Point IIA VI.6.1.1 6.1.1 Acute oral toxicity in rats (LD_{50} test)

		weighed and observed for fourteen days after dosing. [redacted] (wet) is a 73.1% BIT technical grade material. The methodology employed was equivalent to those described in guidelines OECD401, EC B.1.
5.2	**Results and discussion**	There were no deaths and no signs of toxicity at 100 mg/kg and no deaths but signs of slight toxicity at 300 mg/kg. One female dosed with 500 mg/kg was killed *in extremis* and there were signs of slight toxicity in the survivors. Following dosing with 900 mg/kg there were signs of marked toxicity and all the males and three females died or were killed *in extremis* on Days 1 or 2. There were no macroscopic abnormalities in any animal at necropsy. The acute oral median lethal dose value was 670 mg/kg (approximate 95% confidence limits 500, 900) to male rats and 784 mg/kg (lower 95% confidence limit 475) to female rats.
5.3	**Conclusion**	
5.3.1	Reliability	1
5.3.2	Deficiencies	No

Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*August 2008*
Materials and Methods	*Applicant version is adopted*
Results and discussion	*Applicant's version is accepted. It is remarkable that the combined LD_{50} for both sexes is 532 mg/kg bw.*
Conclusion	*According to Annex VI of Directive 67/548/EEC, BIT should be classified as Harmful if swallowed and labelled with phrase R22 and symbol Xn.*
Reliability	*2 (although the study seems to be performed following the ECD guideline 401 and the EC B1 this is not stated in the original Doc. IV).*
Acceptability	*Acceptable*
Remarks	

Table A6_1-1: Table for Acute Toxicity

Dose [mg/kg bw]	Number of dead / number of investigated	Time of death (range)	Observations
100	♂ 0/5 ♀ 0/5	n/a n/a	There were no signs of toxicity at any time in the animals dosed with 100 mg/kg.
300	♂ 0/5 ♀ 0/5	n/a n/a	Signs of slight toxicity in those dosed with 300 mg/kg were piloerection and upward curvature of the spine, neither of which persisted after Day 3.
500	♂ 0/5 ♀ 1/5	n/a Day 2	Surviving animals dosed with 500 mg/kg also showed signs of slight toxicity until Day 3. These were of a slightly higher incidence than those seen following dosing with 300 mg/kg, and included dehydration, piloerection and upward curvature of the spine.
900	♂ 5/5 ♀ 3/5	Days 1-2 Days 1-2	Signs of marked toxicity were observed in the animals dosed with 900 mg/kg. The most common abnormalities were upward curvature of the spine, piloerection, sides pinched-in, dehydration, hypothermia and decreased activity. Two animals appeared cyanosed prior to death. The two surviving females had recovered by days 6 or 7.
LD_{50} value	♂ 670 mg/kg (approximate 95% confidence limits 500, 900) ♀ 784 mg/kg (lower 95% confidence limit 475) Adjusted for 73.1% purity ♂ 490 mg/kg ♀ 573 mg/kg		

Section A6 Toxicological and Metabolic Studies

Subsection A6.1.1/2 Acute Toxicity

Annex Point IIA VI.6.1.1 6.1.1 Acute oral toxicity in rats (LD_{50} test)

			Official use only
		1 REFERENCE	
1.1	**Reference**	[redacted].; 1994; 1,2-Benzisothiazolin-3-one: Acute oral toxicity study in the rat. [redacted]., Report No. 93/NLL044/1051; GLP; Unpublished	
1.2	**Data protection**	Yes	
1.1	Data owner	Clariant Production UK Ltd	
1.2	Companies with letter of access	Arch Chemicals Inc, Thor GmbH	
1.3	Criteria for data protection	Data on existing substance for first entry in to Annex I.	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	Yes OECD 401, EC B.1	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	Batch No. [redacted]	
3.1.2	Specification	As given in section 2	
3.1.2.1	Purity	Not specified	
3.1.2.2	Stability	Not applicable (single administration)	
3.2	**Test Animals**		
3.2.1	Species	Rat	
3.2.2	Strain	CD (remote Sprague-Dawley origin)	
3.2.3	Source	[redacted]	
3.2.4	Sex	♂♀	

Section A6 Toxicological and Metabolic Studies

Subsection A6.1.1/2 Acute Toxicity

Annex Point IIA VI.6.1.1 6.1.1 Acute oral toxicity in rats (LD_{50} test)

3.2.5	Age/weight at study initiation	♂ 102 -138 g (5 weeks old) ♀ 104 -115 g (5 weeks old)
3.2.6	Number of animals per group	♂ 5 ♀ 5 (lowest test concentration only)
3.2.7	Control animals	No
3.3	**Administration/ Exposure**	Oral
3.3.1	Postexposure period	14 days
3.3.2	Type	Gavage
3.3.3	Doses	202, 320 and 506 mg/kg bw
3.3.4	Vehicle	Aqueous 0.5% w/v methylcellulose
3.3.5	Concentration in vehicle	10.1, 16 and 25.3 mg/mL nominal
3.3.6	Total volume applied	20 mL/kg. The dose-volume was calculated for each animal according to its weight at the time of dosing.
3.3.7	Control	No controls included in the experimental design
3.4	**Examinations**	Three separate recordings of signs were made during the first hour after dosing and two further recordings during the remainder of Day 1. From Day 2 onwards, the animals were inspected twice daily and the recordings were made once daily. The circumstances of any death were recorded. The bodyweight of each animal was recorded on the day before dosing and on Days 1, 8 and 15. The test was terminated on the morning of Day 15. Surviving animals were killed at termination of the study. Carcases were stored in a refrigerator at approximately 4°C until trained necropsy staff were available. All animals were thoroughly examined for abnormality of tissues or organs. All body cavities were opened, larger organs were sectioned and the gastro-intestinal tract was opened at intervals for examination of the mucosal surfaces. All abnormalities were described or the normal appearance of major organs was confirmed.

Section A6 Toxicological and Metabolic Studies

Subsection A6.1.1/2 Acute Toxicity

Annex Point IIA VI.6.1.1 6.1.1 Acute oral toxicity in rats (LD_{50} test)

3.5	**Method of determination of LD_{50}**	Probit analysis by the method of Finney (1971) was used to determine the acute median lethal dosage, 95% confidence interval and slope of the dose response curve of the test material.
3.6	**Further remarks**	Only male animals were tested at the three dose levels. Females were included at the lowest dose level to show that females were not more sensitive than male animals. A balance of the calculated amount of test material necessary to prepare the formulations and the quantity actually used was determined. This balance was checked before the formulations were dispensed.
		4 RESULTS AND DISCUSSION
4.1	**Mortality**	Male animals Two male animals treated at 506 mg/kg died within the first hour after dosing and another animal was found dead two hours later. One animal treated at 320 mg/kg died during the first overnight period. There was no death at the low dosage of 202 mg/kg. Ante mortem signs comprised lethargy, underactivity, staggering gait, prone position, pallor, hyperpnoea, piloerection, hunched posture and pupil dilation. Signs of reaction to treatment in the surviving animals in all dose levels included underactivity, staggering gait, piloerection, hunched posture and salivation. The majority of animals were overtly normal five days after dosing. Female animals There was no death and there was no sign of reaction to treatment.
4.2	**Clinical signs**	Necropsy findings for the decedents were unremarkable. Necropsy of the surviving animals, on Day 15, revealed no significant macroscopic lesion.
4.3	**Pathology**	The surviving animals achieved expected bodyweight gains
4.4	**Other**	♂ 454 mg/kg (95% confidence limits 306, 601 mg/kg) ♀ The female animals treated at 202 mg/kg showed no sign of reaction to treatment, indicating the test material was not more toxic to female rats.
4.5	**LD_{50}**	**Male animals** Two male animals treated at 506 mg/kg died within the first hour after dosing and another animal was found dead two hours later. One animal treated at 320 mg/kg died during the first overnight period.

Section A6 Toxicological and Metabolic Studies

Subsection A6.1.1/2 Acute Toxicity

Annex Point IIA VI.6.1.1 6.1.1 Acute oral toxicity in rats (LD_{50} test)

There was no death at the low dosage of 202 mg/kg.

Ante mortem signs comprised lethargy, underactivity, staggering gait, prone position, pallor, hyperpnoea, piloerection, hunched posture and pupil dilation.

Signs of reaction to treatment in the surviving animals in all dose levels included underactivity, staggering gait, piloerection, hunched posture and salivation. The majority of animals were overtly normal five days after dosing.

Female animals

There was no death and there was no sign of reaction to treatment.

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and methods

Young adult rats (CD strain, remote Sprague-Dawley origin) were held in a limited-access facility kept at slight positive pressure relative to the outside. Target values for temperature and humidity were 21°C (range 19-25°C) and 55% R.H. (range 40%-70% R.H.), respectively. A commercially-available complete pelleted rodent diet was fed *ad libitum*.

The main study was carried out using three groups of five male rats (one group at each concentration level) and one group of five female rats (at the lowest dose level). Each group was given a single oral administration of BIT at dosages of 202, 320 or 506 mg/kg, at a constant volume-dosage of 20 mL/kg in aqueous 0.5% w/v methylcellulose.

Animals were inspected regularly and the bodyweight of each animal was recorded on the day before dosing and on Days 1, 8 and 15. The test was terminated on the morning of Day 15.

All animals were examined for abnormality of tissues or organs. All body cavities were opened, larger organs were sectioned and the gastro-intestinal tract was opened at intervals for examination of the mucosal surfaces. All abnormalities were described or the normal appearance of major organs was confirmed.

The methodology employed was as described in guidelines OECD401, EC B.1.

Section A6 Toxicological and Metabolic Studies

Subsection A6.1.1/2 Acute Toxicity

Annex Point IIA VI.6.1.1 6.1.1 Acute oral toxicity in rats (LD_{50} test)

5.2	**Results and discussion**	Two male animals treated at 506 mg/kg died within the first hour after dosing and another animal was found dead two hours later. One animal treated at 320 mg/kg died during the first overnight period. There was no death at the low dosage of 202 mg/kg. Ante mortem signs comprised lethargy, underactivity, staggering gait, prone position, pallor, hyperpnoea, piloerection, hunched posture and pupil dilation. Signs of reaction to treatment in the surviving animals in all dose levels included underactivity, staggering gait, piloerection, hunched posture and salivation. The majority of animals were overtly normal five days after dosing. There was no sign of reaction to treatment in female animals. Necropsy findings for the decedents were unremarkable. Necropsy of the surviving animals, on Day 15, revealed no significant macroscopic lesion. The surviving animals achieved expected bodyweight gains. ♂ LD_{50} = 454 mg/kg (95% confidence limits 306, 601 mg/kg) ♀ The female animals treated at 202 mg/kg showed no sign of reaction to treatment, indicating the test material was not more toxic to female rats.
5.3	**Conclusion**	
5.3.3	Reliability	1
5.3.4	Deficiencies	No

Evaluation by Competent Authorities

EVALUATION BY RAPPORTEUR MEMBER STATE

Date	*August 2008*
Materials and Methods	*Applicant version is accepted.*
Results and discussion	*Applicant version is accepted.*
Conclusion	*According to Annex VI of Directive 67/548/EEC, BIT should be classified as Harmful if swallowed and labelled with phrase R22 and symbol Xn.*

Section A6 Toxicological and Metabolic Studies

Subsection A6.1.1/2 Acute Toxicity

Annex Point IIA VI.6.1.1 6.1.1 Acute oral toxicity in rats (LD_{50} test)

Reliability	*2 (It is assumed a purity of 100% in the assayed material, although this is not stated in the original study. Nevertheless, the study is accepted because the result is comparable to other similar studies presented by the applicant in the dossier).*
Acceptability	*Acceptable.*
Remarks	*Certain mistakes have been detected in the Table A6_1-1:* *a) Animals dosed with 320 mg/kg bw died in the first overnight period and not at day 2;* *b) Animals dosed with 506 mg/kg bw died within the first hour after exposure and not at day 1-2;* *c) Salivation is lost in the lists of signs of reaction to treatment in the surviving animals;* *d) Signs of reaction to treatment in the surviving animals were identical regarding the dose;* *e) Clinical signs ended in surviving animals by day 5-6 and not by day 5 as is stated in animals dosed with 202 and 506 mg/kg bw.*

Table A6_1-1: Table for Acute Toxicity

Dose [mg/kg bw]	Number of dead / number of investigated	Time of death (range)	Observations
202	♂ 0/5 ♀ 0/5	n/a n/a	♂ Signs of reaction to treatment included underactivity, staggering gait, piloerection, hunched posture and salivation. No abnormalities were observed from days 2 – 15. ♀ The female animals showed no sign of reaction to treatment.
320	♂ 1/5	Day 2	♂ The clinical sign of reaction to treatment was piloerection. No clinical signs were observed in surviving animals from days 5 or 6 - 15.
506	♂ 3/5	Days 1-2	♂ Ante mortem clinical signs in decedents comprised lethargy, underactivity, staggering gait, prone position, pallor, hyperpnoea, piloerection, hunched posture and pupil dilation. Signs of reaction to treatment in the surviving animals included underactivity, staggering gait, piloerection and hunched posture. No clinical signs were observed in surviving animals from day 3 - 15.

LD_{50} value	♂ LD_{50} = 454 mg/kg (95% confidence limits 306, 601 mg/kg) ♀ The female animals treated at 202 mg/kg showed no sign of reaction to treatment, indicating the test material was not more toxic to female rats.

Section A6 Toxicological and Metabolic Studies

Subsection A6.1.2/1 Acute Toxicity

Annex Point IIA VI.6.1.2 6.1.2 Acute dermal toxicity in rats (Limit Test)

			Official use only
		1 REFERENCE	
1.1	**Reference**	[redacted] 1988 (reformatted 1990); [redacted] acute dermal toxicity to the rat (Phase 3 Reformat). [redacted] Report No. [redacted]P/2065.	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Arch Chemicals Inc	
1.2.2	Companies with letter of access	Clariant Production UK Ltd, Thor GmbH	
1.2.3	Criteria for data protection	Data on existing substance for first entry in to Annex I.	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	Yes US EPA PAG 81-2 Acute Dermal Toxicity, Rat	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	Reference [redacted]	
3.1.2	Specification	As given in section 2	
3.1.2.1	Purity	73.1% (From [redacted]P/2079)	
3.1.2.2	Stability	Not applicable (single administration)	

Section A6 Toxicological and Metabolic Studies

Subsection A6.1.2/1 Acute Toxicity

Annex Point IIA VI.6.1.2 6.1.2 Acute dermal toxicity in rats (Limit Test)

3.2	**Test Animals**	
3.2.1	Species	Rat
3.2.2	Strain	Wistar-derived albino (Alpk:APfSD)
3.2.3	Source	[redacted]
3.2.4	Sex	♂♀
3.2.5	Age/weight at study initiation	♂ 249-261 g ♀ 180-189 g
3.2.6	Number of animals per group	♂ 5 ♀ 5
3.2.7	Control animals	No
3.3	**Administration/ Exposure**	Dermal – limit test at 2000 mg/kg bw
3.3.1	Postexposure period	14 days

Section A6 Toxicological and Metabolic Studies

Subsection A6.1.2/1 Acute Toxicity

Annex Point IIA VI.6.1.2 6.1.2 Acute dermal toxicity in rats (Limit Test)

3.3.2	Area covered	10 cm × 5 cm, on the dorso-lumbar region
3.3.3	Occlusion	Occlusive
3.3.4	Vehicle	Olive oil
3.3.5	Doses	Approximately 1 g/mL
3.3.6	Total volume applied	The appropriate amount of the test sample was added in 0.3 or 0.5 mL of olive oil, calculated for each animal according to its weight at the time of dosing.
3.3.7	Duration of exposure	24 h
3.3.8	Removal of test substance	At the end of the 24-hour contact period, the dressings were carefully cut, using blunt-tipped scissors, removed and discarded. The skin, at the site of application, was cleansed free of any residual test sample using clean swabs of absorbent cotton wool soaked in clean warm water and was then dried gently with clean tissue paper.
3.4	**Examinations**	The animals were observed for signs of systemic toxicity once between one and four hours after application (only gross abnormalities were noted at this time as the presence of the dressings may have affected the behaviour and movement of the rats) and then once daily for systemic toxicity and skin irritation up to Day 15. The animals were weighed immediately before application of the test sample (Day 1), and on Day 3, Day 4, Day 8 and Day 15.

Section A6 Toxicological and Metabolic Studies

Subsection A6.1.2/1 Acute Toxicity

Annex Point IIA VI.6.1.2 6.1.2 Acute dermal toxicity in rats (Limit Test)

3.5	**Method of determination of LD_{50}**	Not required

4 RESULTS AND DISCUSSION

4.1	**Clinical signs**	None of the animals died after a single dermal application of 2000 mg/kg. Signs of slight toxicity were seen in all animals and these persisted until Days 4-5 in males and 2-3 in females. The abnormalities observed included upward curvature of the spine, signs of urinary incontinence and signs of diarrhoea. It was considered that these abnormalities were probably due to the presence of the occlusive bandage and were therefore not of toxicological significance. Signs of slight skin irritation were observed in all animals and were still present in two animals 14 days after application. These signs included desquamation and erythema. The skin of most animals was stained brown following application. Summary clinical observations are given in Table A6.1.2-1. Initially, most of the animals lost bodyweight slightly. However, by Day 4 all had increased in weight in comparison with their initial (Day 1) value, and continued to do so until the end of the study (Day 15). Individual bodyweights are given in Table A6.1.2-2.
4.2	**Pathology**	There were no macroscopic abnormalities at necropsy.
4.3	**Other**	None
4.4	**LD_{50}**	> 2000 mg/kg bw

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1	**Materials and methods**	[redacted] was assessed for its acute dermal toxicity according to US EPA PAG 81-2, Acute Dermal Toxicity, Rat. One dose level of 2000 mg/kg was used. The test sample was applied to the shorn backs of a group of five male and five female rats, as a paste applied in olive oil. Exposure was maintained for 24 hours under occlusive dressings. At the end of the 24-hour contact period, the dressings were removed and the skin, at the site of application, was cleansed free of any residual test sample using clean swabs of absorbent cotton wool soaked in clean warm water and was then dried gently with clean tissue paper. The rats were observed for fourteen days after dosing.
5.2	**Results and discussion**	None of the animals died after a single dermal application of 2000 mg/kg. The acute dermal median lethal dose was therefore in excess of

Section A6 Toxicological and Metabolic Studies

Subsection A6.1.2/1 Acute Toxicity

Annex Point IIA VI.6.1.2 **6.1.2 Acute dermal toxicity in rats (Limit Test)**

		2000 mg/kg to male and female rats. Signs of slight toxicity were observed following application but these were considered to be due to the occlusive dressing. In addition, all animals showed signs of slight skin irritation which persisted throughout most of the study. There were no macroscopic abnormalities at necropsy. In conclusion, a single dermal application of [redacted] did not produce a toxic effect in rats, but was a slight skin irritant.
5.3	**Conclusion**	
5.3.1	Reliability	1
5.3.2	Deficiencies	No

Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*August 2008*
Materials and Methods	*Applicant version is accepted.*
Results and discussion	*Applicant version is accepted.*
Conclusion	*Doc. IV does not state purity of the test sample and it might be concluded that is treated as a sample of 100% of purity. However, according to this Doc. III the purity of the test material was 73.1%. Therefore, the dose employed in this limit study was 1462 mg BIT/kg bw and it is concluded that the dermal LD_{50} must be higher than 1462 mg BIT/kg bw. In accordance with Council Directive 67/548/EEC, chemicals with dermal LD_{50} for rat or rabbit between 400 and 2000 mg /kg bw must be classified as harmful in contact with skin and labelled with the phrase R21 and the symbol Xn. However, it will be proposed that BIT remains unclassified regarding dermal toxicity on the basis of the following considerations:* *1) none of the animals died during the study,* *2) no abnormalities were present at the pathology examinations;* *3) only minor reversible systemic effects (probably due to the occlusive bondage) were detected; and* *4) only minor irritation effects were recorded.*

Section A6 Toxicological and Metabolic Studies

Subsection A6.1.2/1 Acute Toxicity

Annex Point IIA VI.6.1.2 6.1.2 Acute dermal toxicity in rats (Limit Test)

Reliability	*1*
Acceptability	*Acceptable*
Remarks	

Table A6.1.2-1: Summary clinical observations

	Sex	Clinical observation	Incidence	No. affected /5
Irritation Effects	♂	Skin stained: readable	6	5
		Test substance applied	5	5
		Cracking	1	1
		Clipped	10	5
		Desquamation	39	5
		Applic. Area decontaminated	5	5
		Erythema	3	1
	♀	Skin stained: readable	6	3
		Test substance applied	5	5
		Cracking	1	1
		Clipped	10	5
		Desquamation	37	5
		Applic. Area decontaminated	5	5
		Erythema	6	4
Systemic Effects	♂	Killed termination	5	5
		Diarrhoea	1	1
		Signs of diarrhoea	3	2
		Chromodacryorrhea	1	1

	Sex	Clinical observation	Incidence	No. affected /5
		Signs of urinary incontininence	7	3
		PM examination: NAD	5	5
		Upward curvature of spine	12	5
	♀	Killed termination	5	5
		Diarrhoea	-	-
		Signs of diarrhoea	1	1
		Chromodacryorrhea	-	-
		Signs of urinary incontininence	7	5
		PM examination: NAD	5	5
		Upward curvature of spine	6	5

Table A6.1.2-2: Individual bodyweight data

[redacted]: ACUTE DERMAL TOXICITY TO THE RAT
TABLE 3
INDIVIDUAL BODYWEIGHT DATA (g)

DOSE: 2000 MG/KG

ANIMAL NUMBER	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9	DAY 10	DAY 11	DAY 12	DAY 13	DAY 14	DAY 15
MALES															
22	254	-	257	268	-	-	-	299	-	-	-	-	-	-	335 (D)
23	260	-	257	268	-	-	-	297	-	-	-	-	-	-	345 (D)
24	261	-	255	268	-	-	-	293	-	-	-	-	-	-	344 (D)
25	249	-	246	251	-	-	-	284	-	-	-	-	-	-	323 (D)
26	250	-	244	254	-	-	-	287	-	-	-	-	-	-	324 (D)
MEAN	254.8		251.8	261.8				292.0							334.2
S.D.	5.5		6.3	8.6				6.4							10.5
FEMALES															
27	180	-	178	182	-	-	-	203	-	-	-	-	-	-	226 (D)
28	189	-	188	193	-	-	-	214	-	-	-	-	-	-	238 (D)
29	187	-	193	192	-	-	-	202	-	-	-	-	-	-	230 (D)
30	189	-	190	201	-	-	-	221	-	-	-	-	-	-	246 (D)
31	186	-	186	191	-	-	-	208	-	-	-	-	-	-	240 (D)
MEAN	186.2		187.0	191.8				209.6							236.0
S.D.	3.7		5.7	6.8				8.0							8.0

Section A6	**Toxicological and Metabolic Studies**	
Subsection A6.1.3	**ACUTE INHALATION STUDY**	
Annex Point IIA, 6.1.3		
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ]	**Technically not feasible [ ]** **Scientifically unjustified [ ]**	
Limited exposure [ ]	**Other justification [X]**	
Detailed justification:	**Scientifically unjustified/Limited exposure:** According to the Technical Guidance on data requirements, Ch. 2: Core data set / Part A, 6.1 Acute Toxicity *[Ann IIA, VI. 6.1.]*, "...substances other than gases, shall be administered via at least two routes, one of which should be the oral route. The choice of the second route will depend upon the nature of the substance and the likely route of human exposure. Gases and volatile liquids should be administered by the inhalation route." As neither the pure nor the technical grade active substance is a gas or volatile liquid, and as the nature of the technical grade active substance (a paste) makes the most likely route of exposure dermal, in accordance with the guidance above, the most appropriate second route of administration is dermal. Additionally, according to 6.1.3 Inhalation *[Ann IIA, VI. 6.1.3.]*, "Inhalation toxicity must be reported where the active substance is: - a volatile substance (vapour pressure > 1 x 10^{-2} Pa at 20°C), - a powder containing a significant proportion (e.g. > 1% on a weight basis) of particles with particle size MMAD < 50 micrometers or - to be included in preparations which are powders or are to be applied in a manner which generates aerosols, particles or droplets in the inhalable size range (MMAD < 50 micrometers)." On review; a) 1,2-Benzisothiazol-3-(2*H*)-one is a non-volatile substance with vapour pressure << 1 x 10^{-2} Pa at 20°C (actual value 6.3 × 10^{-5} Pa, *Ann IIA, III. 3.2*), therefore testing is not required on this basis. b) Technical Grade BIT is supplied as a wet paste, which therefore precludes the possibility of spontaneous dust cloud generation. Parr-Dobrzanski (1993) undertook an assessment of the apparent aerodynamic particle size distribution of [redacted] A proportion of the test material was capable of becoming readily airborne (34% less than 115 µm Aerodynamic Equivalent Diameter (AED); the upper particle size cut off of the elutriator). The majority of fractions into which particles were classified had AED's indicating that if suspended in air and inhaled, any material which entered the respiratory tract would deposit in the nasopharyngeal region. From here, clearance to the gastrointestinal tract would be rapid. Only a small proportion of the test material (1.6%) had an AED of < 15µm; particles of this size range being considered capable of penetrating to	

Section A6 **Subsection A6.1.3** **Annex Point IIA, 6.1.3**	**Toxicological and Metabolic Studies** **ACUTE INHALATION STUDY**	
	the lower regions of the respiratory tract. Therefore, it was concluded that [redacted] Press Paste has limited potential for presenting an inhalation hazard from fine particles. c) The active substance is placed on the market as a preparation which is not applied in a manner which generates aerosols, particles or droplets. Furthermore, the biocidal product is exclusively used in industrial settings where inhalation exposure is minimised by the appropriate use of LEV and PPE. **Technically not feasible:** Historically, several attempts have been made to generate an aerosol from the Technical Grade BIT in order to dose in an acute inhalation toxicity test. Because Technical Grade BIT is provided as a wet paste, this aerosol generation for a sustained 4 hour period has proven not possible. **Reference** [redacted].; 1993; [redacted] Assessment of Apparent Aerodynamic Particle Size Distribution by Air Elutriation. [redacted] Report No. [redacted]P/4002; GLP; Unpublished	
Undertaking of intended data submission [ ]	-	
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*July 2021*	
Evaluation of applicant's justification	*Applicant submitted a inhalation toxicity study in 2012 which is attached in this box:* DocIIIA.docx	
Conclusion	*LC_{50} = 0.5 mg BIT/L*	
Remarks		

Section A6 Toxicological and Metabolic Studies

Subsection A6.1.4/1 Acute Dermal Irritation

Annex Point IIA VI.6.1.4 **6.1.4 Acute dermal irritation**

			Official use only
		6 REFERENCE	
6.1	**Reference**	[redacted] 1993; [redacted] skin irritation to the rabbit. [redacted] Report No. [redacted]P/3958; GLP; Unpublished	
6.2	**Data protection**	Yes	
1.2.1	Data owner	Arch Chemicals Inc	
1.2.2	Company with letter of access	Clariant Production UK Ltd, Thor GmbH	
1.2.3	Criteria for data protection	Data on existing substance for first entry in to Annex I.	
		7 GUIDELINES AND QUALITY ASSURANCE	
7.1	**Guideline study**	Yes US EPA PAG 81-5 Acute Dermal Irritation	
7.2	**GLP**	Yes	
7.3	**Deviations**	No	
		8 MATERIALS AND METHODS	
8.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	[redacted]	
3.1.2	Specification	As given in section 2	
3.1.2.1	Purity	74.3%	
3.1.2.2	Stability	Not applicable (single administration)	
8.2	**Test Animals**		
3.2.1	Species	Rabbit	
3.2.2	Strain	New Zealand White albino	
3.2.3	Source	[redacted]	

Section A6 Toxicological and Metabolic Studies

Subsection A6.1.4/1 Acute Dermal Irritation

Annex Point IIA VI.6.1.4 6.1.4 Acute dermal irritation

[redacted]

3.2.4	Sex	Male
3.2.5	Age/weight at study initiation	3587-4825g.
3.2.6	Number of animals per group	6
3.2.7	Control animals	Each animal serves as its own control
8.3	**Administration/ Exposure**	Dermal
3.3.1	Application	
3.3.1.1	Preparation of test substance	500 mg of test substance was moistened with 0.5 mL of deionised water
3.3.1.2	Test site and Preparation of Test Site	Approximately twenty-four hours before application of the test sample, the hair was removed with a pair of veterinary clippers from an area approximately 7 cm × 13 cm on the left flank of each animal, within which the test site area was approximately 2.5 cm x 2.5 cm
3.3.2	Occlusion	Semi-occlusive
3.3.3	Vehicle	Deionised water
3.3.4	Concentration in vehicle	1 g/mL
3.3.5	Total volume applied	0.5 mL
3.3.6	Removal of test substance	Using clean swabs of absorbent cotton wool soaked in clean warm water
3.3.7	Duration of exposure	4 h
3.3.8	Postexposure period	4 days
3.3.9	Controls	Each animal serves as its own control
8.4	**Examinations**	

Section A6 Toxicological and Metabolic Studies

Subsection A6.1.4/1 Acute Dermal Irritation

Annex Point IIA VI.6.1.4 **6.1.4 Acute dermal irritation**

3.4.1	Clinical signs	No
3.4.2	Dermal examination	Yes
3.4.3	Scoring system	Draize
3.4.4	Examination time points	30-60 min, 24 h, 48 h, 72 h, 96 h
3.4.5	Other examinations	None

8.5 Further remarks If necessary, hair growth in test areas was clipped prior to observation

9 RESULTS AND DISCUSSION

9.1 Average score

4.1.1 Erythema

30-60 min	24 h	48 h	72 h	96 h
1.33	1.0	1.0	0.5	0.0*

* Average of 3 scores rather than 6 at 96h

4.1.2 Oedema

30-60 min	24 h	48 h	72 h	96 h
0.83	0.33	0.33	0.17	0.0*

* Average of 3 scores rather than 6 at 96h

9.2 Reversibility Yes

Very slight or well defined erythema was present on the application sites of all animals for up to and including Day 2 or 3.

Very slight oedema was present on 5 of the 6 animals after decontamination. This persisted in 2 animals for a further 2 or 3 days.

9.3 Other examinations None

9.4 Overall result [redacted] was a slight irritant to rabbit skin, following a single four-hour application.

10 APPLICANT'S SUMMARY AND CONCLUSION

10.1 Materials and methods A sample of [redacted] was assessed for its skin irritation potential according to US EPA PAG 81-5.

A group of six male rabbits received a single four-hour application of

Section A6 Toxicological and Metabolic Studies

Subsection A6.1.4/1 Acute Dermal Irritation

Annex Point IIA VI.6.1.4 **6.1.4 Acute dermal irritation**

		500 mg (moistened with 0.5 mL water) of the test sample to the shorn flank. The animals were assessed for up to 4 days for any signs of skin irritation.	
10.2	**Results and discussion**	Following a single four-hour application, very slight or well defined erythema was present on the application sites of all animals for up to and including Day 2 or 3. Very slight oedema was present on 5 of the 6 animals after decontamination. This persisted in 2 animals for a further 2 or 3 days. No other signs of irritation were seen.	
10.3	**Conclusion**	[redacted] was a slight irritant to rabbit skin, following a single four-hour application.	**X**
5.3.1	Reliability	1	
5.3.2	Deficiencies	No	

Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*August 2008*
Materials and Methods	*Applicant's version is accepted.*
Results and discussion	*Applicant's version is accepted.*
Conclusion	*Applicant's conclusion is accepted.*
Reliability	*1*
Acceptability	*Acceptable*
Remarks	*The purity of the test material was 73.1%. Doc. IV states in section 2.4: "The test sample (approximately 500 mg) was moistened with …". Thus, it seems that the dose was prepared without considerations about concentration of active substance (74.3%). Therefore, it might be concluded that the acute dermal irritation study was performed assaying with 372 mg of active substance.*
Date	*May 2020*
Conclusion	*Whilst slight irritation was noted at 60 minutes, by 24 hours the test data demonstrate that BIT should not be classified as a skin irritant and therefore the classification H315 is unwarranted.*

Section A6 Toxicological and Metabolic Studies

Subsection A6.1.4/2 Acute Eye Irritation

Annex Point IIA VI.6.1.4.b/01 **6.1.4 Acute eye irritation (a)**

			Official use only
		1 REFERENCE	
1.1	**Reference**	[redacted] 1993; [redacted] eye irritation to the rabbit. [redacted] Report No. [redacted]P/3961; GLP; Unpublished	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Arch Chemicals Inc	
1.2.2	Company with letter of access	Clariant Production UK Ltd, Thor GmbH	
1.2.3	Criteria for data protection	Data on existing substance for first entry in to Annex I.	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.2	**Guideline study**	Not reported – equivalent to US EPA PAG 81-4 - Acute eye irritation	
2.3	**GLP**	Yes	
2.4	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	[redacted]	
3.1.2	Specification	As given in section 2	
3.1.2.1	Purity	74.3%	
3.1.2.2	Stability	Not applicable (single administration)	
3.2	**Test Animals**		
3.2.1	Species	Rabbit	
3.2.2	Strain	New Zealand White albino	
3.2.3	Source	[redacted]	

3.2.4 Sex	Male
3.2.5 Age/weight at study initiation	3937g for low volume application 4089g for full volume application
3.2.6 Number of animals per group	In accordance with the stepwise approach to ocular irritation assessment, one animal was dosed initially with a low volume application of the test material. As the reaction seen in this animal was less than severe, another animal was dosed with a full volume application of the test material.
3.2.7 Control animals	Each animal serves as its own control
3.3 Administration/ Exposure	
3.3.1 Preparation of test substance	Applied undiluted
3.3.2 Amount of active substance instilled	Low volume: 10 mg Full volume: 100 mg
3.3.3 Exposure period	11 d for low volume application 2.9 h for full volume application
3.3.4 Postexposure period	See above
3.4 Examinations	
3.4.1 Ophthalmoscopic examination	Yes
3.4.2 Scoring system	Immediately after the application of the test sample, an assessment of the initial pain reaction of the rabbits was made using a six-point scale (Table A6.1.4(2)-1). The eyes were examined and the Draize scale (Table A6.1.4(2)-2) was used to assess the grade of ocular reaction at various times. In addition, as an aid in the assessment of corneal damage, fluorescein staining was used.
3.4.3 Examination time points	Refer to individual results Tables A6.1.4(2)-3-4

Section A6 Toxicological and Metabolic Studies

Subsection A6.1.4/2 Acute Eye Irritation

Annex Point IIA VI.6.1.4.b/01 **6.1.4 Acute eye irritation (a)**

3.4.4 Other examinations	Refer to individual results Tables A6.1.4(2)-3-4
3.5 Further remarks	Due to the severity of the irritation response observed the full volume application animal was humanely terminated at 2.9 h.
	4 RESULTS AND DISCUSSION
4.1 Clinical signs	Refer to Tables A6.1.4(2)-3 to 4
4.2 Average score	24, 48, 72 h
4.2.1 Cornea	Low volume: 0 Full volume (2.9 h only): 2
4.2.2 Iris	Low volume: 0 Full volume (2.9 h only): 1
4.2.3 Conjunctiva	
4.2.3.1 Redness	Low volume: 1.66 Full volume (2.9 h only): 3
4.2.3.2 Chemosis	Low volume: 0.66 Full volume (2.9 h only): 4
4.3 Reversibility	Low volume: Yes: All effects reversible by 11 days Full volume (2.9 h only): No
4.4 Other	Initial pain reaction was moderate for both low and full volume.
4.5 Overall result	Due to the severity of the irritation response observed the full volume application animal was humanely terminated. Therefore, 1,2-Benzisothiazol-3-(2*H*)-one should be regarded as at least an extremely severe ocular irritant.
	5 APPLICANT'S SUMMARY AND CONCLUSION
5.1 Materials and methods	A sample of [redacted] was assessed for ocular irritation potential in accordance with a protocol equivalent to US EPA PAG 81-4. In accordance with a stepwise approach to ocular irritation assessment, one animal was dosed initially with a low volume application (10 mg) of the test material. As the reaction seen in this animal was less than severe, another animal was dosed with a full volume application (100 mg) of the test material. Both eyes of each rabbit were examined within the twenty-four hours

prior to the study. The examination consisted of a visual assessment with the aid of fluorescein and only rabbits without any apparent eye defects or ocular irritation were used. Male rabbits were used for this study. The bodyweight of each animal was recorded at the start of the study.

Initially the low volume (10 mg) test sample was applied into the conjunctival sac of the left eye of one rabbit by gently pulling the lower lid away from the eyeball to form a cup into which the test sample was dropped. The lids were then gently held together for 1-2 seconds after which the animal was released. The other eye was untreated (control eye).

Immediately after the application of the test sample, an assessment of the initial pain reaction of the rabbit was made using a six-point scale.

Approximately one week later, a second animal was dosed in a similar manner, with a full volume (100 mg) application of the test material, and the initial pain reaction was also noted.

The eyes were examined and the Draize scale was used to assess the grade of ocular reaction. In addition, as an aid in the assessment of corneal damage, fluorescein staining was used.

5.2 Results and discussion

Low Volume (10 mg)

Application of the test material caused moderate initial pain (class 3 on a scale of 0-5) to the rabbit eye.

No corneal or iridial effects were observed. Conjunctival effects included slight to moderate redness, slight to mild chemosis and slight discharge, which persisted up to and including Day 4. Other signs of irritation seen included erythema and dried secretions on the eyelids, mucoid discharge, haemorrhaging of the nictitating membrane and superficial erosion of the corneal epithelium. The animal also held its dosed eye partially closed on the day of dosing, indicating that it was experiencing pain.

Full Volume (100 mg)

Application of the test material caused moderate initial pain (class 3 on a scale of 0-5) to the rabbit eye. A small amount of the test material was not dosed due to the level of pain experienced by the animal.

Corneal and iridial effects could not be assessed due to severe swelling of the conjunctival sac. Other conjunctival effects included severe redness and severe discharge. After approximately 3 hours the degree of conjunctival swelling had increased and indeed lead to rupture of the conjunctival sac; the severe redness and discharge were still also evident. At this point the animal was removed from the study and humanely terminated. Immediately after the animal was killed, the corneal and iridial scores were read. This revealed mild corneal opacity affecting the whole cornea and slight iritis. Other signs of irritation seen included erythema on the eyelids, haemorrhaging of the

Section A6 Toxicological and Metabolic Studies

Subsection A6.1.4/2 Acute Eye Irritation

Annex Point IIA VI.6.1.4.b/01 **6.1.4 Acute eye irritation (a)**

		nictitating membrane and constricted pupil. Red staining (probably blood) was also seen around the dosed eye.
5.3	**Conclusion**	Due to the severity of the irritation response observed in the full volume application, the animal was humanely terminated. Therefore, 1,2-Benzisothiazol-3-(2*H*)-one should be regarded as at least an extremely severe ocular irritant.
4.2.1	Reliability	1
4.2.2	Deficiencies	No

Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*August 2008*
Materials and Methods	*Applicant's version is accepted.*
Results and discussion	*Applicant's version is accepted.*
Conclusion	*Applicant's version is accepted. It is also remarkable that according to Annex VI of Directive 67/548/EEC, BIT should be labelled with R41 (risk of serious damage to the eyes) and with the symbol Xi (irritant).*
Reliability	*2 (although the study seems to be performed following the USEPA guideline 81-4 this is not stated in the original Doc. IV).*
Acceptability	*Acceptable*
Remarks	*The purity of the test material was 74.3%. Doc. IV states that "the test sample was applied into the sac …". Thus, it seems that the dose was prepared without considerations about concentration of active substance (74.3%).* *Therefore, it might be concluded that the main acute eye irritation study was performed assaying with mg 74 mg of active substance.*

Table A6_1_4(2)-1: Initial pain evaluation

Class	Reactions by Animal	Descriptive Rating
0	No response.	No initial pain.
1	A few blinks only normal within one or two minutes.	Practically no initial pain.
2	Rabbit blinks and tries to open eye, but the reflexes close it.	Slight.initial pain.
3	Rabbit holds eye shut and puts pressure on lids; may rub eye with paw.	Moderate initial pain.
4	Rabbit holds eye shut vigorously; may squeal.	Severe initial pain.
5	Rabbit holds eye shut vigorously; may squeal, claw at eye, jump and try to escape.	Very severe initial pain.

Table A6_1_4(2)-2.: Scoring (Draize, 1959)

	Descriptive Rating
1. CORNEA	
(a) Opacity - Degree of Density (area most dense taken for reading)	
No opacity	0 none
Scattered or diffuse area, details of iris clearly visible	1 slight
Easily discernible translucent areas, details of iris slightly obscured	2 mild
Opalescent areas, no details of iris visible, size of pupil barely discernible	3 moderate
Opaque, iris invisible	4 severe
(b) Area of Cornea Involved	
One quarter (or less) but not zero	1
Greater than one quarter, but less than half	2
Greater than half, but less than three quarters	3
Greater than three quarters, up to whole area	4
a x b x 5 total maximum	80
2. IRIS	
(a) Values	
Normal	0 none
Folds above normal, congestion, swelling, circumcorneal injection (any or all of these or combination of any thereof) iris still reacting to light, (sluggish reaction is positive)	1 slight
No reaction to light, haemorrhage, gross destruction (any or all of these)	2 severe
a x 5 Total maximum	10
3. CONJUNCTIVAE	
(a) Redness (refers to palpebral and bulbar conjunctivae excluding cornea and iris)	
Vessels normal	0 none
Vessels definitely injected above normal	1 slight
More diffuse, deeper crimson red, individual vessels not easily discernible	2 moderate
Diffuse beefy red	3 severe
(b) Chemosis	
No swelling	0 none
Any swelling above normal (includes nictitating membrane)	1 slight
Obvious swelling with partial eversion of lids	2 mild
Swelling with lids half-closed	3 moderate
Swelling with lids about half-closed to completely closed	4 severe
(c) Discharge	
No discharge	0 none
Any amount different from normal (does not include small amounts observed in inner canthus of normal animals)	1 slight
Discharge with moistening of the lids and hairs just adjacent to lids	2 moderate
Discharge with moistening of the lids and hairs, and considerable area around the eye	3 severe
(a + b + c) x 2 Total maximum	20

Table A6_1_4(2)-3: Results of eye irritation study (Low volume – Single animal)

score	Cornea	Iris	Conjunctiva			Other observations
			redness	chemosis	discharge	
	0 to 4	0 to 2	0 to 3	0 to4	0 to 3	
1.3 h	0	0	1	2	1	Eye closed partially (pain?) (5 h)
24 h	0	0	2	1	1	Fluorescein staining
48 h	0	0	2	1	1	Slight discharge: mucoid
72 h	0	0	1	0	0	Slight erythema of the upper and/or lower eyelids Dried secretions around the eyelids Nictitating membrane partially haemorrhagic
96 h	0	0	1	0	0	Dried secretions around the eyelids Nictitating membrane partially haemorrhagic
11 days	0	0	0	0	0	-
Average 24h, 48h, 72h	0	0	1.66	0.66	0.66	-
Area effected	0	-	-	-	-	-
Reversibility*	c	c	c	c	c	-
average time for reversion	n/a	n/a	11 d	72 h	72 h	-

* c : completely reversible
n c : not completely reversible
n : not reversible

Table A6_1_4(2)-4: Results of eye irritation study (High volume – Single animal)

score	Cornea	Iris	Conjunctiva			Other observations
			redness	chemosis	discharge	
	0 to 4	0 to 2	0 to 3	0 to4	0 to 3	
1.5 h	?	?	3	4	3	Test substance in conjunctival sac Slight erythema of the upper and/or lower eyelids Peri orbital area stained pink
2.9 h	2	1	3	4	3	Test substance in conjunctival sac Slight erythema of the upper and/or lower eyelids Nictitating membrane partially haemorrhagic Conjunctiva partially haemorrhagic Pupil constriction Blood stained discharge? Animal terminated prior to final observation
Area effected	4	-	-	-	-	-
Reversibility*	n	n	n	n	n	-
average time for reversion	n/a	n/a	n/a	n/a	n/a	-

* c : completely reversible
n c : not completely reversible
n : not reversible
? unable to assess due to chemosis

Section A6 Toxicological and Metabolic Studies

Subsection A6.1.5/1 SKIN SENSITISATION

Annex Point IIA VI.6.1.5/01 **Magnusson-Kligman method**

			Official use only
		1 REFERENCE	
1.1 Reference		[redacted] 1990; Benzisothiazolin-3-one: skin sensitisation to the Guinea Pig. [redacted] Report No. [redacted]P/3101; GLP; Unpublished	
1.2 Data protection		Yes	
1.2.1	Data owner	Arch Chemicals Inc	
1.2.2	Companies with letter of access	Clariant Production UK Ltd, Thor GmbH	
1.2.3	Criteria for data protection	Data on existing substance for first entry in to Annex I.	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1 Guideline study		Not reported – equivalent to; US EPA PAG 81-6 - Skin sensitisation	
2.2 GLP		Yes	
2.3 Deviations		No	
		3 MATERIALS AND METHODS	
3.1 Test material		As given in section 2	
3.1.1	Lot/Batch number	[redacted]	
3.1.2	Specification	Deviating from specification given in section 2 as follows The Test Substance employed was pre-dried technical grade active substance	
3.1.2.1	Description	Not reported	
3.1.2.2	Purity	Not applicable (repeat acute administration)	

Section A6 Toxicological and Metabolic Studies

Subsection A6.1.5/1 SKIN SENSITISATION

Annex Point IIA VI.6.1.5/01 **Magnusson-Kligman method**

3.1.2.3	Stability	for induction:	subcutaneously injected as a 0.01% w/v preparation in 3% w/v dimethylformamide (DMF) in corn oil and as a 0.01% w/v preparation in a 1:1 Freund's Complete Adjuvant plus 3% w/v DMF in corn oil and topically applied as a 30% w/v preparation in DMF.
		for challenge:	topically applied as a 10% w/v preparation in DMF and as a 3% w/v preparation in DMF.
3.1.2.4	Preparation of test substance for application	Yes	
3.2	**Test Animals**		
3.2.1	Species	Guinea pig	
3.2.2	Strain	Alpk:Dunkin Hartley albino	
3.2.3	Source	[redacted]	
3.2.4	Sex	Female	
3.2.5	Age/weight at study initiation	Young adults weighing 313-368g (main study)	
3.2.6	Number of animals per group	20 test group, 10 negative control group, 20 positive control group	
3.2.7	Control animals	Negative control and positive control	
3.3	**Administration/ Exposure**	State study type: Adjuvant	
3.3.1	Induction schedule	Intradermal: Day 0 Topical: Day 7	
3.3.2	Way of induction	Intradermal and topical Occlusive	

Section A6 Toxicological and Metabolic Studies

Subsection A6.1.5/1 SKIN SENSITISATION

Annex Point IIA VI.6.1.5/01 **Magnusson-Kligman method**

3.3.3	Concentrations used for induction	Intradermal: A row of three injections (0.05-0.l mL each) was made on each side of the mid-line. The injections were: i) Freund's Complete Adjuvant plus 3% w/v DMF in corn oil in the ratio 1:1; ii) 0.01% (w/v) preparation of the test sample in 3% w/v DMF in corn oil; iii) 0.01% (w/v) preparation of the test sample in a 1:1 Freund's Complete Adjuvant plus 3% w/v DMF in corn oil Topical: One week later, the scapular area was clipped again and treated with a topical application of the test sample as a 30% (w/v) preparation in DMF. Induction of the control animals: intradermal injections were administered using an identical procedure to that used for the test animals, except that the injections were: (i) Freund's Complete Adjuvant plus 3% w/v DMF in corn oil in the ratio 1:1 (ii) 3% w/v DMF in corn oil only (iii) Freund's Complete Adjuvant plus 3% w/v DMF in corn oil in the ratio 1:1 The topical applications followed the same procedure as for the test animals except that DMF only was applied to the filter paper.
3.3.4	Concentration Freunds Complete Adjuvant (FCA)	FCA + 3% w/v DMF in corn oil in the ratio 1:1
3.3.5	Challenge schedule	Day 21
3.3.6	Concentrations used for challenge	10% (w/v) in DMF 3% (w/v) in DMF
3.3.7	Rechallenge	No
3.3.8	Scoring schedule	24 h and 48 h after removal of the dressing
3.3.9	Removal of the test substance	After twenty four hours, the dressings were carefully cut, using blunt-tipped scisssors, removed and discarded. There is no record of removal of the test substance from the skin.

3.3.10	Positive control substance	Formaldehyde
3.4 Examinations		24 and 48 hours after removal of the dressing, any erythematous reactions were quantified using the four-point scale shown below and the number of positive responses was recorded. 0 - no reaction 1 - scattered mild redness 2 - moderate diffuse redness 3 -intense redness and swelling
3.4.1	Pilot study	yes
3.5 Further remarks		

4 RESULTS AND DISCUSSION

4.1 Results of pilot studies		The dose levels for each of the three stages of the main study were determined by a sighting study in which groups of two guinea pigs were used and up to five dose-levels were tested on each group of animals. The procedure was as follows; i) intradermal injection (induction): preparations of the test sample in 3% w/v DMF in corn oil were tested to determine the highest concentration, up to 0.01% w/v, that could be well tolerated locally and systemically; ii) topical application (induction): preparations of the test sample in DMF were tested to determine the highest concentration which did not cause greater than a mild to moderate irritation response, in animals that had been injected with Freund's Complete Adjuvant at least fourteen days previously; iii) topical application (challenge): preparations of the test sample in DMF were tested to determine the highest concentration which did not produce irritation in animals that had been injected with Freund's Complete Adjuvant at least fourteen days previously. The dosing in the main study was based upon the results of the pilot study.
4.2 Results of test		
4.2.1	24 h after challenge	High dose (10% w/v): 13/20 Low dose (3% w/v): 2/20

4.2.2	48 h after challenge	High dose (10% w/v): 13/20 Low dose (3% w/v): 1/20
4.2.3	Other findings	Positive control results: Following challenge with a 50% (w/v) dilution of the 40% (w/v) aqueous formaldehyde solution, scattered mild redness to moderate diffuse redness was seen in fourteen out of the seventeen test animals scored. No response was seen in any of the ten control animals. The net percentage response was calculated to be 82%.
4.3 Overall result		Following challenge with a 10% (w/v) preparation of the test sample in DMF, scattered mild redness to intense redness and swelling was seen in thirteen out of twenty test animals. Scattered mild redness was seen in three out of ten control animals. The net percentage response was calculated to be 35%. Following challenge with a 3% (w/v) preparation of the test sample in DMF, scattered mild redness was seen in two out of twenty test animals. No erythematous response was seen in any of the control animals. The net percentage response was calculated to be 10%.

% net response	description
0	not a sensitiser
1-8	weak sensitiser
9-28	mild sensitiser
29-64	moderate sensitiser
65-80	strong sensitiser
81-100	extreme sensitiser

5 APPLICANT'S SUMMARY AND CONCLUSION

Section A6 Toxicological and Metabolic Studies

Subsection A6.1.5/1 SKIN SENSITISATION

Annex Point IIA VI.6.1.5/01 **Magnusson-Kligman method**

5.1 Materials and methods

The sensitising properties of the test sample were assessed using a method based on the maximisation test of Magnusson and Kligman (1970).

(a) Induction

The hair was removed from an area approximately 5cm x 5cm on the scapular region of each animal with a pair of veterinary clippers and a row of three injections (0.05-0.l mL each) was made on each side of the mid-line. The injections were:

i) Top: Freund's Complete Adjuvant plus 3% w/v DMF in corn oil in the ratio 1:1;

ii) Middle: a 0.01% (w/v) preparation of the test sample in 3% w/v DMF in corn oil;

iii) Bottom: a 0.01% (w/v) preparation of the test sample in a 1:1 Freund's Complete Adjuvant plus 3% w/v DMF in corn oil.

The injections were checked for any adverse effects for up to 48 hours.

One week later, the scapular area was clipped again and treated with a topical application of the test sample as a 30% (w/v) preparation in DMF. This preparation (0.2-0.3 mL) was applied on filter paper held in place by surgical tape. The tape was covered by an occlusive dressing which was kept in place for 48 hours.

The application sites were checked approximately 24 hours after removal of the dressings.

(b) Challenge

Two weeks after the topical inductions, an area, approximately 15cm x 5cm, on both flanks of all the test and control animals, was clipped free of hair with a pair of veterinary clippers. An occlusive dressing was prepared which consisted of two pieces of filter paper stitched to a piece of rubber sheeting.

A 10% (w/v) preparation of the test sample (0.05-0.l mL) in DMF was applied to one of the pieces of filter paper and a 3% (w/v) preparation in DMF (0.05-0.l mL) was applied to the second piece of filter paper. The dressing was placed on to the guinea pig so that the 10% (w/v) preparation was on the left shorn flank and the 3% (w/v) preparation was on the right shorn flank. It was then covered with a strip of adhesive bandage which was secured by a self-adhesive PVC tape.

After twenty four hours, the dressings were removed and discarded.

After a further 24 and 48 hours, any erythematous reactions were quantified and the number of positive responses was recorded.

Section A6 Toxicological and Metabolic Studies

Subsection A6.1.5/1 SKIN SENSITISATION

Annex Point IIA VI.6.1.5/01 **Magnusson-Kligman method**

5.2 Results and discussion

Challenge of previously induced guinea pigs with a 10% (w/v) preparation of benzisothiazolin-3-one in DMF elicited a moderate skin sensitisation response and challenge with a 3% (w/v) preparation elicited a mild skin sensitisation response.

Therefore, benzisothiazolin-3-one was a moderate skin sensitiser under the conditions of the test.

5.3 Conclusion

5.3.1 Reliability 1

5.3.2 Deficiencies No

Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*August 2008*
Materials and Methods	*Applicant's version is accepted.*
Results and discussion	*Applicant's version is accepted*
Conclusion	*In accordance to Annex VI of Directive 67/548/EEC, BIT should be labeled with phrase R43 (may cause sensitization by dermal contact) and the symbol Xi (irritant).*
Reliability	*2 (although the study seems to be performed following the guideline USEPA 81-6 this is not stated in the original Doc. IV; see also comments in section of remarks).*
Acceptability	*Acceptable*
Remarks	*Purity of the active ingredient is reported neither in this document nor in the corresponding Doc. IV. Nevertheless, the study is considered as acceptable because despite the unknown purity of BIT, it caused sensitization.*

Section A6 Toxicological and Metabolic Studies

Subsection A6.2/1 Absorption, distribution, metabolism and excretion

Annex Point IIA VI.6.2.a/01 **TOXICOKINETIC**

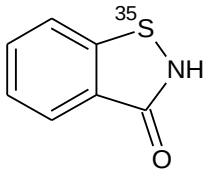
		Official use only
	1 REFERENCE	
1.1 Reference	[redacted] 1976; 1,2-Benzisothiazolin-3-one: Metabolism in the Rat and Dog. [redacted] Report No. [redacted]P/227; GLP; Unpublished	
1.2 Data protection	Yes	
1.2.1 Data owner	Arch Chemicals Inc	
1.2.2 Companies with letter of access	Clariant Production UK Ltd, Thor GmbH	
1.2.3 Criteria for data protection	Data on existing substance for first entry in to Annex I.	
	2 GUIDELINES AND QUALITY ASSURANCE	
2.1 Guideline study	No No specific guidelines were followed for this study as it was designed to provide preliminary data to allow the comparison of the metabolism of [^{35}S]-BIT following oral administration in rats and beagle dogs.	
2.2 GLP	No GLP was not compulsory at the time the study was performed	
2.3 Deviations	No No guidelines followed	
	3 MATERIALS AND METHODS	
3.1 Test material	Radiolabelled BIT	
3.1.1 Lot/Batch number	Not reported	
3.1.2 Specification	Deviating from specification given in section 2 as follows: The test material is radiolabelled.	
3.1.2.1 Description	Specific radioactivity 1.3 mCi mmole^{-1}	

Section A6 Toxicological and Metabolic Studies

Subsection A6.2/1 Absorption, distribution, metabolism and excretion

Annex Point IIA VI.6.2.a/01 **TOXICOKINETIC**

3.1.2.2	Purity	100% radiopurity [based on the statement that only a single radioactive component was detected when the compound was chromatographed on thin-layer plates of silica gel C in three solvent systems]
3.1.2.3	Stability	Not applicable (single administration)
3.1.2.4	Radiolabeling	Radiolabelled with Sulphur-35



3.2 Test animals

3.2.1	Species	Rat and Dog
3.2.2	Strain	Rat: Wistar Dog: Beagle
3.2.3	Source	In-house
3.2.4	Sex	Rat: ♂ Dog: ♂
3.2.5	Age/weight at study initiation	Rat: 200 g Dog: 15.5 kg
3.2.6	Number of animals	Rat: 10 (oral) 2 (intraperitoneal) Dog: 1 (oral)
3.2.7	Control animals	No
3.3	**Administration/ Exposure**	Rat: Oral and intraperitoneal Dog: Oral
3.3.1	Animals and dosing	Ten male rats were dosed orally with [^{35}S]-BIT (400 mg/kg; 0.23 µCi). The animals were housed collectively and the 24-hour combined urine sample was retained. Two male rats were given an intraperitonea1 dose of [^{35}S]-BIT (2.2 µCi). The animals were transferred to individual metabolism cages for the separate collection of urine.

		One Beagle dog was given a gelatin capsule containing [^{35}S]-BIT (1.2 mg/kg; 4.6 µCi) in corn oil. The animal was housed in an individual metabolism cage and was fed twice daily.
3.3.2	Measurement of radioactivity	Radioactivity of all samples was determined using a Packard Tricarb 3002 Liquid Scintillation Spectrometer. Counting efficiency was determined using a solution of [^{35}S]-BIT as an internal standard. As the same solution was used for dosing, this obviated the need for making any correction for the radiochemical decay of the isotope.
3.3.3	Fractionation and purification of urine	Pooled 24-hour urine from animals given [^{35}S]-BIT was freeze-dried and triturated with ethyl acetate. The solution was filtered, evaporated under reduced pressure at 49°C and the residue dissolved in the minimum volume of water. The solution was adjusted to pH with saturated sodium bicarbonate and extracted with ethyl acetate. The solvent was evaporated and the residue dissolved in methanol.
3.3.4	Chromatography	Solutions of metabolites were applied to thin-layer plates (5 x 20 cm and 20 x 20 cm : 500 µ) of silica gel G and developed one- or two-dimensionally with one of the following solvent systems: (A) *n*-Butanol: ethanol: water (10:2.3:v/v) (B) *n*-Butanol saturated with water (C) *n*-Butanol: glacial acetic acid: water (4:l:5:v/v:upper phase) (D) *n*-Butanol saturated with 2N ammonia (E) Chloroform: methanol (9:l:v/v) (F) Methanol: ethyl acetate (l:l:v/v) (G) Benzene: methanol (l:l:v/v) (H) Isopropanol: ammonia (0.88): water (90:1:9:v/v) Metabolites were detected by viewing under UV light (254 nm), while radioactive areas were located either by a radio-chromatogram scan, or by radio-autography using Kodak X-ray film. After development, the relative distribution of metabolites was assessed by removing and counting areas of support corresponding to radioactivity. Individual metabolites eluted from the support with methanol were further purified where necessary by chromatography on plates of silica gel HR (500 µ). Purified metabolites were characterised by co-chromatography with the available authentic compound and were analysed by mass spectrometry.
3.3.5	Enzymic hydrolysis of [^{35}S]-BIT metabolites	Solutions of metabolites were taken to dryness, redissolved in 0.2M sodium acetate buffer (10 mL pH 4.5) and incubated for 18 hours at 37°C with β-glucuronidase (2000 units) containing aryl sulphatase. The incubation mixture was evaporated to dryness and triturated with methanol. The methanol extracts were compared by chromatography with

those from a control incubation without enzyme.

3.3.6 Determination of [^{35}S]-sulphate ion in rat urine

Pooled 24-hour urine (25 mL) from rats given [^{35}S]-BIT was diluted to 50 mL with water. Sodium sulphate (1 g) was added, the solution was acidified with hydrochloric acid and an excess of barium chloride solution (0.05 N) was added. The precipitate was collected, washed with water and acetone and dried in a vacuum desiccator, Portions of this residue (100 mg) were determined for radioactivity.

4 RESULTS AND DISCUSSION

4.1 Toxic effects, clinical signs

None reported

4.2 Recovery of labelled compound

Not reported; however, Conning (1972) and O'Connor (1999) have shown that > 96% of an oral dose of BIT is excreted by rats in five days and that neither BIT nor any of its metabolites are retained or accumulated in the liver or abdominal fat.

4.3 Metabolism study

Two-dimensional chromatography showed that three metabolites were present, coded metabolites 1, 2 and 3.

Mass spectrometry of metabolite 2 showed the parent ion occurring at ^{m}/e 183.0355 which corresponds to $C_8H_9NO_2S$. A peak also occurred at 168.0118 $(M\text{-}CH_3)^+$. This indicated that metabolite 2 was o-(methyl-sulphinyl)benzamide. When examined by mass spectrometry, metabolite 3 gave the parent ion at ^{m}/e 199.0301 ($C_8H_9SO_3N$). This suggested that metabolite 3 was o-(methylsulphonyl) benzamide. The identity of metabolites 2 and 3 was confirmed by co-chromatography with the authentic compounds in solvent systems (D), (E), (G) and (H).

The identity of metabolite 1 was not determined, however the compound did not co-chromatograph with o-(methylthio)benzamide.

The relative distribution of metabolites in rat and dog urine is given in Table A6_2(2)-1. A metabolic pathway is postulated in Figure A6_2-1.

Chromatography in solvent systems (A)-(H) indicated that β-glucuronidase/ aryl sulphatase did not cause any hydrolysis.

Measurements of radioactivity indicated that a negligible amount of sulphate ion was present in the rat urine.

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and methods

Ten male rats were dosed orally with [^{35}S]-BIT (400 mg/kg; 0.23 µCi). The animals were housed collectively and the 24-hour combined urine sample was retained.

Two male rats were given an intraperitonea1 dose of [^{35}S]-BIT alone (2.2 µCi). The animals were transferred to individual metabolism cages for the

separate collection of urine.

One Beagle dog was given a gelatin capsule containing [^{35}S]-BIT (1.2 mg/kg; 4.6 µCi) in corn oil. The animal was housed in an individual metabolism cage and was fed twice daily.

Radioactivity of all samples was determined. Pooled 24-hour urine from animals given [^{35}S]-BIT was freeze-dried and triturated with ethyl acetate. The solution was filtered, evaporated under reduced pressure at 49°C and the residue dissolved in the minimum volume of water. The solution was adjusted to pH with saturated sodium bicarbonate and extracted with ethyl acetate. The solvent was evaporated and the residue dissolved in methanol.

Solutions of metabolites were applied to thin-layer plates of silica gel and developed one- or two- dimensionally.

Purified metabolites were characterised by co-chromatography with the available authentic compound and were analysed by mass spectrometry.

5.2 Results and discussion

Summarize relevant results; discuss dose-response relationship.

Two-dimensional chromatography showed that three metabolites were present, coded metabolites 1, 2 and 3.

Mass spectrometry of metabolite 2 showed the parent ion occurring at ^{m}/e 183.0355 which corresponds to $C_8H_9NO_2S$. A peak also occurred at 168.0118 $(M\text{-}CH_3)^+$. This indicated that metabolite 2 was *o*-(methyl-sulphinyl)benzamide. When examined by mass spectrometry, metabolite 3 gave the parent ion at ^{m}/e 199.0301 ($C_8H_9SO_3N$). This suggested that metabolite 3 was *o*-(methylsulphonyl) benzamide. The identity of metabolites 2 and 3 was confirmed by co-chromatography with the authentic compounds in solvent systems (D), (E), (G) and (H).

The identity of metabolite 1 was not determined, however the compound did not co-chromatograph with *o*-(methylthio)benzamide.

The relative distribution of metabolites in rat and dog urine is given in Table A6_2(2)-1. A metabolic pathway is postulated in Figure A6_2-1.

Chromatography in solvent systems (A)-(H) indicated that β-glucuronidase/ aryl sulphatase did not cause any hydrolysis.

Measurements of radioactivity indicated that a negligible amount of sulphate ion was present in the rat urine.

5.3 Conclusion

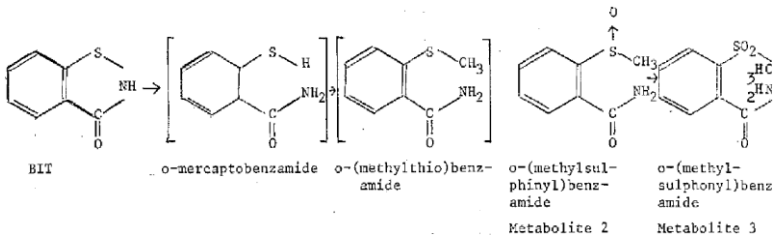
The routes of metabolism of BIT in the dog and rat are essentially similar. The breakdown of BIT by both species is rapid and is carried virtually to completion, since no unchanged BIT was found in either dog or rat urine. BIT does not appear to persist in the body since Conning (1972) found that neither BIT nor any of its metabolites are retained or accumulated in the liver or abdominal fat.

Williams (1959) has described the metabolic breakdown of disulphides, which may be considered to be similar to the compound under

investigation. Disulphides are usually reduced in the body to mercaptans, the general metabolic reaction being

$R.S.S.R^1 \rightarrow RSH+R^1SH$

Williams also states that methylation of these SH compounds to yield sulphides which could then be oxidised to sulpones, is a possible further reaction. Thus, a possible route of metabolic breakdown for 1,2-benzisothiazolin-3-one is as follows:-



This route is in agreement with that suggested by Palmer and Jones (1973) for the biodegradation of BIT in an aqueous system after the addition of an inoculum derived from raw settled sewage.

5.3.1 Reliability

Based on the assessment of materials and methods include appropriate reliability indicator 0, 1, 2, 3, or 4

2

5.3.2 Deficiencies

Yes

Recovery of labelled compound was not reported, however, Conning (1972) and O'Connor (1999) have shown that > 96% of an oral dose of BIT is excreted by rats in five days and that neither BIT nor any of its metabolites are retained or accumulated in the liver or abdominal fat.

Evaluation by Competent Authorities

EVALUATION BY RAPPORTEUR MEMBER STATE

Date *August 2008*

Materials and Methods *Applicant version is accepted.*

Results and discussion *Applicant version is accepted.*

Conclusion *Applicant's conclusion is adopted.*

Section A6 Toxicological and Metabolic Studies

Subsection A6.2/1 Absorption, distribution, metabolism and excretion

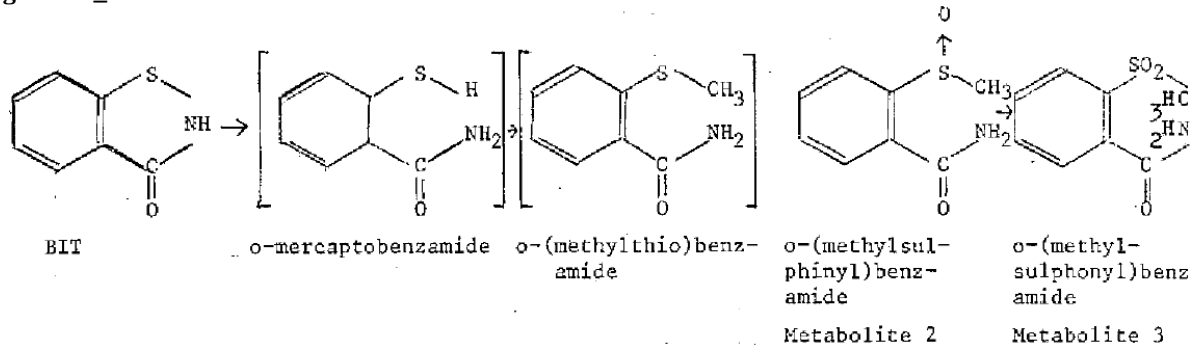
Annex Point IIA VI.6.2.a/01 **TOXICOKINETIC**

Reliability	*2 (No specific guideline, no GLP).*
Acceptability	*Acceptable*
Remarks	

Table A6_2(2)-1: Relative distribution of metabolites in rat and dog urine

Animal species	Route	Metabolites expressed as percentage of radioactivity in Day 1 urine		
		1	2	3
Rat	Oral	25	66	9
Rat	Intraperitoneal	7	81	12
Dog	Oral	12	68	19

Figure A6_2-1: Possible Route of Metabolic Breakdown of BIT



Section A6 Toxicological and Metabolic Studies

Subsection A6.2/2 Percutaneous absorption (*in-vivo* test)

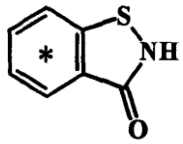
Annex Point IIA
VI.6.2.b/01

		Official use only
1 REFERENCE		
1.1 Reference	[redacted] 1999; [redacted] BIT: Preliminary Study to Assess the Absorption of [redacted] BIT from the Skin and Gastrointestinal Tract in the Male Rat. [redacted]., Report No. NLL060/970078; GLP; Unpublished	
1.2 Data protection	Yes	
1.2.1 Data owner	Clariant Production UK Ltd	
1.2.2 Company with letter of access	Arch Chemicals Inc, Thor GmbH	
1.2.3 Criteria for data protection	Data on existing substance for first entry in to Annex I.	
2 GUIDELINES AND QUALITY ASSURANCE		
2.1 Guideline study	No No specific guidelines were followed for this study as it was designed to provide preliminary data to allow the comparison of the absorption of [^{14}C]-[redacted] BIT following oral administration and topical application.	
2.2 GLP / GCP	Yes	
2.3 Deviations	No No guidelines followed	
3 MATERIALS AND METHODS		
3.1 Test material	Radiolabelled BIT	
3.1.1 Lot/Batch number	Radiolabelled BIT: [redacted] Non-radiolabelled BIT: [redacted]	
3.1.2 Specification	Deviating from specification given in section 2 as follows: The test material is radiolabelled.	
3.1.2.1 Purity	Radiolabelled BIT: > 98%	

Non-radiolabelled BIT: 96.8%

3.1.2.2 Stability — Not applicable (single administration)

3.1.2.3 Radiolabelling — 1 ,2-[benzene-U-^{14}C]-Benzisothiazolin-3-one



3.2 Test animals

3.2.1 Species — Rat

3.2.2 Strain — Sprague Dawley

3.2.3 Source — [redacted]

3.2.4 Sex — Male

3.2.5 Age/weight at study initiation — 5-7 weeks/180-200 g

3.2.6 Number of animals per group — 8 animals

Group No.	Dose route	Study Section	Animal Nos.
1	Oral	Tissue Distribution	1-5
1	Oral	Whole Body Autoradiography (WBA)	6-8
2	Dermal	Tissue Distribution	9-13
2	Dermal	Whole Body Autoradiography (WBA)	14-16

3.2.7 Control animals — No

3.3 Administration/ Exposure

3.3.1 Preparation of test — **Oral dose (Group 1)**

site

The formulations were administered by oral gavage using a syringe and cannula. The dose for each animal was aspirated volumetrically into a pre-weighed syringe with an attached rubber cannula, which was then re-weighed. At regular intervals between doses, quality control checks were taken by diluting a known weight of the dose formulation (approximately 0.1 g) with a known weight of acetone (approximately 20 g).

Dermal dose (Group 2)

An area of dorsal skin was shaved on the day prior to dosing. Immediately prior to dosing a silicone rubber saddle was attached to the shaved area using cyanoacrylate adhesive. At dosing, the formulation was applied via a pipette (calibrated to dispense 120 µL) to an area of shaved skin approximately 12 cm^2. The dose was then evenly distributed over the selected area using a dose spreader which was retained, rinsed with acetone and analysed to determine the residual dose.

Following administration the application site was semi-occluded with a stainless steel gauze held in place over the silicone rubber saddle by surgical tape.

3.3.2 Concentration of test substance

The doses were formulated to deliver the test material as a single oral or dermal dose at 10 mg/kg and 5 MBq/kg.

A known weight of radiolabelled material was dissolved in a volume of acetone and quantified by radiochemical analysis. Portions of this stock solution were thoroughly mixed with non-radiolabelled material (previously dissolved in acetone). The acetone was removed, under a gentle stream of nitrogen, and the residue re-suspended in propylene glycol, sonicated briefly and left stirring until fully dissolved. When fully dissolved, glycerol and distilled water were added to the propylene glycol in the following ratio:

Proplyene glycol : Glycerol : Distilled water (45:30:25 by volume).

Formulation components	Volume formulation component (mL)	
	Oral administration	Topical application
Proplyene glycol	18	1.73
Glycerol	12	1.15
Distilled water	10	0.96

3.3.3 Specific activity of test substance

Radiolabelled BIT: Spec. Act. 199 µCi/mg (7360 kBq/mg)

3.3.4 Volume applied

Oral dose (Group 1): 2 mL

		Dermal dose (Group 2): 10 µL/cm^2
3.3.5	Size of test site	Oral dose (Group 1): Not applicable Dermal dose (Group 2): 12 cm^2
3.3.6	Exposure period	Oral dose (Group 1): 4, 8*, 24*, 48 and 72* hours (tissue distribution, *WBA also) Dermal dose (Group 2): 4, 8*, 24*, 48 and 72* hours (tissue distribution, *WBA also)
3.3.7	Sampling time	**Oral dose (Group 1)** Five of these animals were analysed for tissue residues and three were analysed by WBA. For tissue residues one animal was sacrificed at 4, 8, 24, 48 and 72 hours following dosing. The remaining animals were sacrificed at 8, 24 and 72 hours for WBA. **Dermal dose (Group 2)** Five of these animals were analysed for tissue residues and three were analysed by WBA. For tissue residues one animal was sacrificed at 4, 8, 24, 48 and 72 hours following dosing. The remaining animals were sacrificed at 8, 24 and 72 hours for WBA.
3.3.8	Samples	**Oral dose (Group 1)** Samples were taken to allow tracking of the excretion of radioactive material in the urine, faeces, expired carbon dioxide and volatile organic material. For the animals analysed for tissue residues, under halothane anaesthesia, a sample of blood was removed from a retro-orbital sinus and the rats killed by an overdose of halothane. At sacrifice, the following tissues were removed and placed in individual pre-weighed containers (Animals 1 - 5 only): Carcass intestinal tract and contents kidney liver skin stomach and contents **Dermal dose (Group 2)** Samples were taken to allow tracking of the excretion of radioactive material in the urine, faeces, expired carbon dioxide and volatile organic material. For the animals analysed for tissue residues, under

halothane anaesthesia, a sample of blood was removed from a retro-orbital sinus and the rats killed by an overdose of halothane. At sacrifice, the following tissues were removed and placed in individual pre-weighed containers (Animals 9 - 13 only):

Carcass

intestinal tract and contents

kidney

liver

stomach and contents

treated skin (also collected from animals 14-16)

untreated skin

4 RESULTS AND DISCUSSION

4.1 Toxic effects, clinical signs

No effects

4.2 Dermal irritation

No effects

4.3 Recovery of labelled compound

Oral dose (Group 1)

The overall recovery of radioactive material was 100 ± 2.0% (Animals 1-5 see Table A6_2(1)-1).

Dermal dose (Group 2)

The overall recovery of radioactive material was 106± 1.6% (Animals 9-13 see A6_2(1)-2).

4.4 Percutaneous absorption

Following a single topical application of [^{14}C]-[redacted] BIT (10 mg/kg; 5 MBq/kg) to the shaved dorsal area (12 cm^2) of the male rat the absorption of radioactive material was high. Approximately 40% of the applied radioactivity was absorbed over 72 hours, with a further 47.6% either adsorbed onto or absorbed into the treated skin, and therefore potentially available for absorption following the final collection (72 hours). Of the absorbed radioactive material, 40.6% was excreted over 72 hours (97.6% of the total absorbed material) indicating that rapid excretion and low tissue disposition occurs.

see A6_2(1)-3.

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and methods

The absorption, distribution and excretion of radiolabelled material was determined at 4, 8, 24, 48 and 72 hours following a single oral administration or a single topical application of [^{14}C]-[redacted] BIT (approximately 10 mg/kg; 5 MBq/kg). Any unabsorbed material was

removed from the site by washing with 1% Tween 80 following the final collection.

Animals were sacrificed for quantitative whole body autoradiography at 8, 24 and 72 hours following both oral administration and topical application.

5.2 Results and discussion

The overall recoveries of radioactive material were 100 ± 2.0% for orally dosed animals and 106 ± 1.6% for topically dosed animals.

At 8 hours after a topical application, 3.2% of the radiochemical dose was absorbed and 23.9% remained in the treated skin and was, therefore, available for absorption.

At 72 hours after a topical application, 41.6% of the radiochemical dose was absorbed and 47.6% remained in the treated skin and was, therefore, available for absorption.

Radioactive material was rapidly and extensively absorbed through the skin and from the gastrointestinal tract. At 8 hours after an oral administration, 96.6% of the radiochemical dose was detected in samples other than the gastrointestinal tract. At 72 hours after a topical application, 41.6% of the radiochemical dose was absorbed and 47.6% remained in the treated skin and was, therefore, available for absorption.

The primary route of excretion was in the urine, with 96.6% of the activity absorbed following topical application and 99.5% of the radiochemical dose following oral administration, being excreted by this route in 72 hours.

Very little material was detected in the faeces, indicating that the majority of the radioactive material is absorbed following oral administration and that biliary excretion is unlikely to occur to any great extent (low levels of radioactive material were detected in the faeces and gastrointestinal tract (less than 0.5% of the radiochemical dose combined) following topical application).

The test material does not appear to be broken down to volatile components or excreted in the expired carbon dioxide, as indicated by high overall recoveries and low trap levels (less than 0.05% of the radiochemical dose) of radioactivity in trapping solutions.

Tissue disposition does not appear to occur. Less than 0.05% of the radiochemical dose remained in any tissue at 48 hours after oral administration and topical application, with the exception of the carcass and untreated skin following topical application which, combined, contained less than 1.5% of the radiochemical dose.

5.3 Conclusion

[^{14}C]-[redacted] BIT is rapidly and extensively absorbed from the gastrointestinal tract and through the skin, and is then rapidly excreted, primarily in the urine, with little or no tissue disposition. Low levels of radioactivity were detected in the faeces, indicating that

	the majority of the radioactivity is absorbed following oral administration and that biliary excretion is unlikely to occur (low gastrointestinal tract and faeces levels following dermal application). The test material is not broken down into volatile components or expired as carbon dioxide.
5.3.1 Reliability	1
5.3.2 Deficiencies	No

Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*August 2008*
Materials and Methods	*Applican'st version is accepted.*
Results and discussion	*Applicant's version is accepted.*
Conclusion	*Applicant's conclusion is adopted.*
Reliability	*2 (no specific guideline was followed in the study as their main aim was to provide preliminary information about dermal and oral absorption; see also comments in section of remarks)*
Acceptability	*Acceptable*
Remarks	*The study do not fit the requirements for metabolic and toxicokinetics studies of the Technical Guidance document in support in support of Directive 98/8 where is stated that, usually a study with two single application test (high dose and low dose) plus a study with a repeated dose are required.*

Table A6_2(1)-1 : Recovery of radioactivity from male rats up to 72 hours following a single oral administration of ^{14}C-[redacted] BIT (approximately 10 mg/kg; 5 MBq/kg). Results are expressed as mean percent radiochemical dose.

Animal Number		1M	2M	3M	4M	5M	6M	7M	8M	Mean	SD
Achieved	kBq	1205	1190	1248	1340	1248	1254	1232	1261	1247	44.9
Dose	mg	2.30	2.27	2.38	2.56	2.38	2.39	2.35	2.41	2.38	0.1
Urine	0-4h	44.2	53.0	41.6	40.5	28.2	33.5	22.0	39.3	37.8	9.7
	4-8h	-	30.8	33.8	32.3	33.8	21.1	43.3	27.9	31.9	6.7
	8-24h	-	-	14.0	15.1	29.5	-	21.4	20.7	20.1	6.2
	24-48h	-	-	-	0.9	1.0	-	-	0.9	0.9	0.1
	48-72h	-	-	-	-	0.1	-	-	0.3	0.2	-
TOTAL		44.2	83.8	89.4	88.8	92.6	54.6	86.7	89.1	NA	-
Faeces	0-4h	NS	NS	NS	0.0	NS	NS	NS	NS	0.0	-
	4-8h	-	NS	NS	0.0	NS	NS	NS	NS	0.0	-
	8-24h	-	-	0.6	0.5	1.0	-	0.8	0.8	0.7	0.2
	24-48h	-	-	-	0.3	0.3	-	-	0.9	0.5	0.3
	48-72h	-	-	-	-	0.1	-	-	0.1	0.1	-
TOTAL		0.0	0.0	0.6	0.8	1.4	0.0	0.8	1.8	NA	-
Cage wash	0-4h	4.2	5.7	3.5	3.3	4.0	5.3	3.6	6.8	4.6	1.3
	4-8h	-	1.0	5.2	2.9	1.8	4.0	2.8	2.9	2.9	1.4
	8-24h	-	-	1.2	0.5	1.0	-	1.1	0.7	0.9	0.3
	24-48h	-	-	-	0.1	0.1	-	-	0.1	0.1	0.0
	48-72h	-	-	-	-	0.1	-	-	0.1	0.1	-
TOTAL		4.2	6.7	9.9	6.8	7.0	9.3	7.5	10.6	NA	-
Carbon dioxide	0-4h	ND	ND	ND	ND	ND	ND	ND	ND	ND	-
	4-8h	-	ND	ND	ND	ND	ND	ND	ND	ND	-
	8-24h	-	-	ND	ND	ND	-	ND	ND	ND	-
	24-48h	-	-	-	ND	ND	-	-	ND	ND	-
	48-72h	-	-	-	-	ND	-	-	ND	ND	-
TOTAL		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	NA	-
Volatile Organics	0-4h	ND	ND	ND	ND	ND	ND	ND	ND	ND	-
	4-8h	-	ND	ND	ND	ND	ND	ND	ND	ND	-
	8-24h	-	-	ND	ND	ND	-	ND	ND	ND	-
	24-48h	-	-	-	ND	ND	-	-	ND	ND	-
	48-72h	-	-	-	-	ND	-	-	ND	ND	-
TOTAL		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	NA	-
Liver		2.3	0.4	0.0	0.0	0.0	-	-	-	NA	-
Kidney		0.6	0.1	0.0	0.0	0.0	-	-	-	NA	-
Stomach + contents		9.1	2.9	0.2	0.0	0.0	-	-	-	NA	-
G.I.Tract + contents		6.5	1.6	0.5	0.0	0.0	-	-	-	NA	-
Carcass		26.4	4.5	0.3	0.1	0.0	-	-	-	NA	-
Skin		5.3	1.1	0.3	0.3	0.1	-	-	-	NA	-
TOTAL TISSUES		50.2	10.6	1.3	0.4	0.1				NA	-
OVERALL TOTAL		98.6	101	101	96.8	101	63.9	95.0	102	100*	2.0

The results are expressed to three significant figures or one decimal place.
0.0 - Results < 0.05% of applied radioactivity.
ND -Results within the background range.
NS- No sample.
NA - Not applicable as totals calculated over different collection periods.
* Animals 1-5 only, as there was no tissue analysis for animals 6-8.

Table A6_2(1)-2: Recovery of radioactivity from male rats up to 72 hours following a single dermal administration of ^{14}C-[redacted] BIT (approximately 10 mg/kg; 5 MBq/kg). Results are expressed as mean percent radiochemical dose.

Animal Number		9M	10M	11M	12M	13M	14M	15M	16M	Mean	SD
Achieved	kBq	1351	1352	1360	1361	1356	1360	1356	1359	1357	3.8
Dose	mg	2.48	2.48	2.50	2.50	2.49	2.50	2.49	2.50	2.49	0.0
Urine	0-4h	0.5	0.3	0.3	1.3	1.0	1.5	1.0	1.2	0.9	0.5
	4-8h	-	1.1	1.9	3.3	3.3	1.7	2.5	4.9	2.7	1.3
	8-24h	-	-	11.8	14.2	14.3	-	21.1	18.0	15.9	3.7
	24-48h	-	-	-	14.0	14.0	-	-	19.5	15.8	3.2
	48-72h	-	-	-	-	4.5	-	-	7.1	5.8	-
TOTAL		0.5	1.4	14.0	32.8	37.1	3.2	24.6	50.7	NA	-
Faeces	0-4h	ND	0.0	ND	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	4-8h	-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	8-24h	-	-	0.0	0.0	0.1	-	0.0	0.0	0.0	0.0
	24-48h	-	-	-	0.1	0.0	-	-	0.1	0.1	0.1
	48-72h	-	-	-	-	0.2	-	-	0.3	0.3	-
TOTAL		0.0	0.0	0.0	0.1	0.3	0.0	0.0	0.4	NA	-
Cage wash	0-4h	0.1	0.0	0.0	0.2	0.5	0.3	0.1	0.2	0.2	0.2
	4-8h	-	0.1	0.1	0.4	0.6	0.1	0.2	0.3	0.3	0.2
	8-24h	-	-	0.4	0.7	0.5	-	0.9	1.0	0.7	0.3
	24-48h	-	-	-	1.1	1.0	-	-	0.8	1.0	0.2
	48-72h	-	-	-	-	0.6	-	-	0.6	0.6	-
TOTAL		0.1	0.1	0.5	2.4	3.2	0.4	1.2	2.9	NA	-
Carbon dioxide	0-4h	ND	0.0	ND	ND	ND	ND	ND	ND	0.0	0.0
	4-8h	-	ND	ND	0.0	ND	0.0	0.0	ND	0.0	0.0
	8-24h	-	-	ND	ND	ND	-	ND	ND	ND	-
	24-48h	-	-	-	ND	0.0	-	-	ND	0.0	0.0
	48-72h	-	-	-	-	0.0	-	-	0.0	0.0	-
TOTAL		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	NA	-
Volatile Organics	0-4h	ND	0.0	ND	ND	ND	0.0	0.0	ND	0.0	0.0
	4-8h	-	ND	0.0	ND	ND	0.0	ND	ND	0.0	0.0
	8-24h	-	-	ND	ND	0.0	-	ND	0.0	0.0	0.0
	24-48h	-	-	-	ND	ND	-	-	ND	ND	-
	48-72h	-	-	-	-	0.0	-	-	0.0	0.0	-
TOTAL		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	NA	-
Skin swab		80.1	75.5	46.9	20.8	13.7	62.0	36.8	10.3	NA	-
Gauze wash		7.6	4.5	5.4	1.1	2.1	7.2	0.7	1.9	NA	-
TOTAL		87.7	80.0	52.3	21.9	15.8	69.2	37.5	12.2	NA	-
Liver		0.1	0.1	0.2	0.1	0.0	-	-	-	NA	-
Kidney		0.0	0.0	0.0	0.0	0.0	-	-	-	NA	-
Stomach + contents		0.0	0.0	0.0	0.0	0.0	-	-	-	NA	-
G.I.Tract + contents		0.1	0.1	0.2	0.1	0.1	-	-	-	NA	-
Carcass		0.8	1.2	3.6	0.9	0.4	-	-	-	NA	-
Skin (untreated)		0.1	0.3	0.6	0.5	0.5	-	-	-	NA	-
Skin (treated)		15.1	23.9	36.8	48.7	47.6	26.0	38.7	39.5	NA	-
TOTAL TISSUES		16.2	25.6	41.4	50.3	48.6	26.0	38.7	39.5	NA	-
OVERALL TOTAL		105	107	108	108	105	98.8	102	106	106*	1.6

The results are expressed to three significant figures or one decimal place.
0.0 - Results < 0.05% of applied radioactivity.
ND -Results within the background range.
NA - Not applicable as totals calculated over different collection periods.
* Animals 9-13 only, as there was no tissue analysis for animals 14-16.

Table A6_2(1)-3 : Percentage of radiochemical material and mg equivalents of [redacted] BIT absorbed, excreted, remaining in the treated skin and removed from the site of application following a single topical application of ^{14}C-[redacted] BIT (approximately 10 mg/kg, 5 MBq/kg).

Time (hr)	Animal No.	Dose	Absorbed (1)		Excreted (2)		Treated Skin		Skin Swab/ Gauze Wash		Total
		mg	%	mg	%	mg	%	mg	%	mg	%
4	9	2.48	1.7	0.042	0.6	0.015	15.1	0.374	87.7	2.175	105
8	10	2.48	3.2	0.079	1.5	0.037	23.9	0.593	80.0	1.984	107
24	11	2.50	19.1	0.478	14.5	0.363	36.8	0.920	52.3	1.308	108
48	12	2.50	36.9	0.923	35.3	0.883	48.7	1.218	21.9	0.548	108
72	13	2.49	41.6	1.036	40.6	1.001	47.6	1.185	15.8	0.393	105

(1): Absorbed material is material present in the urine, faeces, cage washes and tissues.

(2): Excreted material is material present in the urine, faeces and cage washes.

Section A6 Toxicological and Metabolic Studies

Subsection A6.3.1 **REPEATED DOSE TOXICITY**

Annex Point IIA, VI.6.3 **28 Day Rat Oral Gavage**

			Official use only
		1 REFERENCE	
1.1	**Reference**	[redacted] 1997; [redacted] BIT: Toxicity Study By Oral Gavage Administration To CD Rats For 4 Weeks. [redacted], Report No. 96/NLL059/1118	
1.2	**Data protection**	Yes	
3.1.1	Data owner	Clariant Production UK Ltd	
3.1.2	Companies with a letter of access	Arch Chemicals Inc and Thor GmbH	
3.1.3	Criteria for data protection	Data on existing substance for first entry in to Annex I.	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	Yes OECD 407	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	[redacted]	
3.1.2	Specification	As given in section 2	
3.1.2.1	Purity	The certificate of analysis received with [redacted] BIT, Batch [redacted] states an organic purity of 99.8%, although the analysis was done on a batch sample, not the specific sample submitted for toxicity testing. Upon receipt of the test item by the testing laboratory the BIT purity of Batch [redacted] was determined as 91.5% by HPLC with UV detection. Consequently, all dosages and concentrations were expressed using a conversion factor of 1.092.	
3.1.2.2	Stability	The homogeneity of distribution and stability of [redacted] BIT in 0.5% methylcellulose were assessed at concentrations of 1.5, 5.0 and 15.0 mg/mL prepared before the commencement of treatment. Six unit dose samples, evenly spaced throughout the bulk preparation were assayed to determine the homogeneity of the formulation and provide	

		the initial concentration value of the stability test. Further samples were assayed after 24 and 48 hours storage at ambient temperature to determine the stability of [redacted] BIT in the formulations. The concentrations of test substance were also determined in formulations prepared for one occasion of dosing during the first and last weeks of treatment. Analysis was performed using HPLC with UV detection. Acceptable homogeneity of the formulation was demonstrated for each concentration and [redacted] BIT was shown to be stable in 0.5% methylcellulose for at least six hours. The concentrations of the analysed preparations from the first and last weeks of treatment were also satisfactory.Gavage formulations were prepared freshly each day, and the formulation allowed to stand for 30 minutes before sampling or dosing. [redacted] BIT was shown to be stable in 0.5% methylcellulose for at least 6 hours.
3.2	**Test Animals**	
3.1.1	Species	Rat
3.1.2	Strain	CD
3.1.3	Source	[redacted]
3.1.4	Sex	Male and female
3.1.5	Age/weight at study initiation	28 to 35 days/96 to 119 g
3.1.6	Number of animals per group	10 animals per group
3.1.7	Control animals	1 vehicle control group
3.3	**Administration/ Exposure**	Oral
3.3.1	Duration of treatment	28 Days
3.3.2	Frequency of exposure	Once per day, 7 days per week.
3.3.3	Postexposure period	There was no post exposure period. Animals were sacrificed after the last dose and there was no post-last dose data collected.
3.3.4	Oral	
3.3.4.1	Type	Gavage

Section A6 Toxicological and Metabolic Studies

Subsection A6.3.1 **REPEATED DOSE TOXICITY**

Annex Point IIA, VI.6.3 **28 Day Rat Oral Gavage**

3.3.4.2	Concentration	15, 50 and 150 mg/kg/day
3.3.4.3	Vehicle	0.5% methylcellulose
3.3.4.4	Concentration in vehicle	1.5, 5.0 and 15.0 mg/mL
3.3.4.5	Total volume applied	10 mL/kg bodyweight
3.3.4.6	Controls	Vehicle controls

3.4 Examinations

3.4.1 Observations

3.4.1.1 Clinical signs

Individual Observations

Individual observations were performed daily during week 1 and twice weekly during weeks 2, 3 and 4. All animals were observed prior to dosing, on return to the cage, at the end of dosing of each group, 1-2 hours after completion of dosing of all groups and as late as possible in the working day. The timing of these observations were performed to establish and confirm any pattern of signs

Functional Observation Battery

Each animal was subjected to the procedures detailed below on the specified occasions. The functional observation battery was performed at the same time of day on each occasion. The procedures were performed by an observer who was unaware of the treatment group to which each animal belonged. Before commencement of each set of observations, the cage labels were covered so that the only information visible to the observer was the animal and cage numbers. The animal and cage numbers did not equate to the standard numbering system used at this laboratory.

Open Field Observations

Before commencement of treatment and during each week of treatment, at the same time of day, the appearance and behaviour of each animal was assessed. The animal was placed on a clean sheet of absorbent paper in an observation arena. The behaviour during a two minute period was observed and recorded in an area of the test room where visual and auditory stimuli were minimised. The following parameters were assessed and graded:

activity, alertness, behaviour, convulsion, defaecation (number and consistency), exophthalmos (abnormal protrusion of the eyes), fur appearance, gait, grooming frequency, lachrimation, palpebral closure (eyelid closure), piloerection (raised fur), posture and pupil size,

removal from cage, respiration rate, salivation, tremor and urination.

Sensory Reactivity Test

During Week 4 of treatment the functional integrity of each animal was assessed using a series of manipulation observations, the observations comprised:

auditory pinna reflex, auditory startle reflex, body temperature recorded using a digital thermometer, flexor (withdrawal) responses, atypically strong responses, landing footsplay, pain (tail pinch) response, pupil closure response, reaction to handling, righting reflex.

Grip Strength and Motor Activity

Also during week 4 of treatment, immediately following the sensory reactivity tests, grip strength and motor activity were assessed.

The forelimb and hindlimb grip strength were measured by an automated grip strength meter. Three trials were performed.

Each animal was placed in a clear polycarbonate cage with eight infra-red beams (four high and four low) to measure both rearing and cage floor activity levels. Activity measurements were recorded for ten six-minute periods (a total of one hour).

Statistical Analysis

For motor activity (each time intervals and total scores), body temperature, grip strength and landing foot splay, homogeneity of variance and normality were assessed by Bartlett's test and Shapiro-Wilk statistic, respectively. If either of the above tests was significant at 0.05 (Bartlett's) or 0.01 (Shapiro-Wilk), between-group significance was assessed by Kruskal-Wallis test.

If the Kruskal-Wallis test was not significant, no further analysis were performed. If the Kruskal-Wallis test was significant at 0.05 level, pair-wise differences (against Control) were assessed by the Wilcoxon Rank Sum test.

If both Bartlett's test and Shapiro-Wilk statistic were not significant, between-group significance was assessed by a one-way analysis of variance (ANOVA). If the ANOVA was not significant, no further analysis were required. If the ANOVA was significant at 0.05 level, pair-wise differences (against Control) were assessed by Dunnett's test.

Group Observations

Animals and the cage-trays were inspected at least twice daily for evidence of reaction to treatment or ill-health. Any deviations from normal were recorded at the time in respect of nature and severity, date and time of onset, duration and progress of the observed condition, as appropriate.

Section A6 Toxicological and Metabolic Studies

Subsection A6.3.1 **REPEATED DOSE TOXICITY**

Annex Point IIA, VI.6.3 **28 Day Rat Oral Gavage**

3.4.1.2	Mortality	Animals were inspected at least twice daily for evidence of reaction to treatment (including mortality).
3.4.2	Body weight	The bodyweight of each animal was recorded during the acclimatisation period, on the day that treatment commenced, weekly intervals throughout the treatment period and before necropsy. The animals were weighed more frequently, when appropriate, for animals displaying ill-health, so that the progress of the observed condition would be monitored.
3.4.3	Food consumption	The weight of food supplied to each cage, that remaining and an estimate of the amount spilled was recorded for each week throughout the treatment period. From these records the mean weekly consumption per animal was calculated for each cage. Group mean food conversion efficiencies were calculated for each week of treatment. Food conversion efficiency is the efficiency of conversion of food to new body tissue. The weekly group mean values were calculated from unrounded weekly cage values (calculated from the bodyweight gain of animals alive at the end of the week and the total food consumed in the cage). Allowance was made for the food consumed by any animal that was killed during the week. Overall group mean values were calculated from the overall bodyweight gain, divided by the total food consumed, expressed as a percentage.
3.4.4	Water consumption	Not recorded
3.4.5	Ophthalmoscopic examination	Not performed
3.4.6	Haematology	<u>Haematology (peripheral blood)</u> During Week 4 of treatment, after completion of open field observations, sensory reactivity test and grip strength and motor activity, blood samples were obtained from all animals, after overnight starvation (before dosing). Blood samples were withdrawn from the retro-orbital sinus, with the animals held under halothane/nitrous oxide anaesthesia, and collected into EDTA as anticoagulant. The samples were obtained and analysed in the sequence Group 1, 4, 2 and 3 for males and females. All samples were examined using a Technicon UHI haematology analyser for the following characteristics: • Packed cell volume • Haemoglobin concentration • Erythrocyte count

- Mean cell haemoglobin concentration
- Mean cell haemoglobin
- Mean cell volume
- Total and differential leucocyte count
- Platelet count

The equipment distinguishes neutrophils, lymphocytes, eosinophils, basophils, monocytes and a small proportion of large unstained cells.

A blood film was prepared in Romanowsky stain and examined by light microscopy for abnormal morphology and unusual cell types, including normoblasts.

Additional samples were taken into citrate anticoagulant and examined in respect of prothrombin time.

Haematology (bone marrow)

Bone marrow samples were obtained from the femur at the necropsy of all animals. Smears from these samples were air-dried, fixed in methanol and stained using a May-Grunwald-Giemsa procedure.

The smears from all animals of Groups 1 and 4 sacrificed on completion of the scheduled treatment period, were examined by counting 100 nucleated cells and computing the myeloid : erythroid ratio. The cellularity and composition of the marrow were also assessed.

Statistics

The significance of inter-group differences in haematology was assessed by Student's t-test using a pooled error variance. Statistical significances for eosinophil, basophil, monocyte and large unstained cell counts are not reported as these data are not normally distributed.

3.4.7 Clinical Chemistry

At the same time as for peripheral haematology further blood samples were taken and collected into lithium heparin as anticoagulant. Samples were taken and analysed in the same sequence as for peripheral haematology. After separation the plasma was examined in respect of:

- Alkaline phosphatase activity
- Alanine amino-transferase activity
- Aspartate amino-transferase activity
- Gamma-glutamyl transpeptidase activity
- Urea concentration
- Glucose concentration

- Total cholesterol concentration
- Creatinine concentration
- Total protein concentration
- Albumin concentration
- Albumin/globulin ratio - calculated from total protein concentration and chemically analysed albumin concentration
- Sodium and potassium - by indirect ion selective electrode

Statistics

The significance of inter-group differences in blood chemistry was assessed by Student's t-test using a pooled error variance.

3.4.8 Urinalysis: Not performed

3.5 Sacrifice and pathology

3.5.1 Organ Weights: The following organs, taken from each animal, were dissected free of adjacent fat and other contiguous tissue and the weights recorded.

Adrenals, liver, brain, spleen, epididymides, testes, heart, thymus and kidneys.

3.5.2 Gross and histopathology:

Tissues Preserved for Histopathology

The following tissue samples were preserved in 4% neutral buffered formaldehyde, except the testes and epididymides which were initially placed in Bouins fluid.

Adrenals, brain, caecum, colon, duodenum, epididymides, heart, ileum, jejunum, kidneys, liver, lungs with mainstem bronchi, lymph nodes (mandibular and mesenteric), ovaries, prostate, sciatic nerve (one only), spinal cord, spleen, stomach, testes, thymus, thyroid with parathyroids, trachea, urinary bladder and uterus with cervix.

Femoral bone marrow smears were processed and examined as detailed in Section 3.4.6.

Samples of any other abnormal tissues were also retained for histopathological examination.

Histology

The tissue samples listed below from the animals specified were dehydrated, embedded in paraffin wax, sectioned at approximately 5 µm thickness and stained with haematoxylin and eosin. For bilateral organs, both sections were prepared and examined.

Adrenals - cortex and medulla

Brain - cerebellum, cerebral cortex and medulla

Heart - auricular and ventricular

Kidneys - cortex, medulla and papilla

Liver - section from each of the left and median lobes

Lungs - section from each of the left and right caudal lobes

Spinal cord - transverse section at the cervical, thoracic and lumbar levels

Stomach- keratinised and glandular

Thyroid - included parathyroids in section

Uterus - uterus section separate from cervix section.

Microscopic examination was performed as follows:

i) Tissues were examined for all animals from Group 1 and 4.

ii) The kidneys, liver, lungs and stomach were examined for all animals from group 2 and 3

iii) Tissues reported at macroscopic examination as being abnormal were examined for all animals.

3.5.3 Other examinations: Not applicable

3.5.4 Statistics: For organ weights and bodyweight changes, homogeneity of variance was tested using Bartlett's test. Whenever this was found to be statistically significant a Behrens-Fisher test was used to perform pairwise comparisons, otherwise a Dunnett's test was used.

Inter-group differences in macroscopic pathology and histopathology were assessed using Fisher's Exact test.

Unless stated, group mean values or incidences for the treated groups were not significantly different from those of the Controls ($p > 0.05$).

Due to the small number of animals in each group, the results of these tests cannot be considered definitive and are used merely as a guide in the interpretation of the results.

3.6 Further remarks: None

4 RESULTS AND DISCUSSION

4.1 Observations

4.1.1 Clinical signs

<u>Individual and Group Observations</u>

Salivation immediately after dosing was observed on most days during the study in animals receiving 150 mg/kg/day. Occasionally, in a few animals the salivation was seen before dosing. In addition, a few

animals receiving 50 mg/kg/day occasionally salivated immediately after dosing.

From the second week of treatment, males receiving 150 mg/kg/day showed hunched posture. A female receiving this dosage was similarly affected during Week 2.

There were no other signs of reaction to treatment. Respiratory noise after dosing and gasping were transient signs in two females receiving 150 mg/kg/day. These signs could not, with any confidence, be attributed to treatment.

Functional Observation Battery

Open Field Observations

The only open field observation considered to be related to treatment was the moderate or marked salivation seen in animals receiving 150 mg/kg/day, which was often accompanied by chin or muzzle rubbing onto the floor of the open field and unusual 'snaking' body movements, possibly representing the animals' attempts to wipe away excess saliva. These salivation and/or chin wiping observations were recorded for the majority of males and a single female at Week 2 and for one male and one female at Weeks 3 and 4: they were considered to be a response to handling and the animals' anticipation of oral dosing.

Compared with controls, urination in the open field appeared to have increased at Week 4 in males receiving 150 mg/kg/day but females were unaffected and it would seem unlikely that this was a response to treatment of any toxicological significance.

Sensory reactivity tests

Body temperature and sensory reactivity responses were considered to have been unaffected by treatment.

Group mean landing footsplay measurements for both males and females receiving 150 mg/kg/day were less than those of controls but the differences were not statistically significant ($p > 0.05$) and it was concluded, after examination of the individual values, that these inter-group differences were due to the naturally high variation often seen in these recordings.

Grip Strength and Motor Activity

Forelimb and hindlimb grip strength values showed some inter-group variation but there was no indication of any effect of treatment.

Motor activity scores for males and females during Week 4 of treatment were essentially similar in all groups.

4.1.2 Mortality

A female receiving 150 mg/kg/day was killed *in extremis* on Day 11 of treatment. Ante mortem signs comprised piloerection, hunched posture and respiratory problems. The respiratory problems suggested

		that a portion of the administered dose may have entered the trachea, though this was not confirmed at macroscopic or histopathological examination. Macroscopic changes included distension of the gastro-intestinal tract and thickening of the keratinised wall of the stomach and histopathologically there was ulceration, hyperkeratosis, epithelial hyperplasia and chronic inflammation of the keratinised stomach and mucous cell hypertrophy in the glandular region. None of these changes was considered to have caused the death of this animal. This death was probably unrelated to treatment.
4.2	**Body weight gain**	Males receiving 150 mg/kg/day gained slightly less weight than Controls. This difference was, however, not statistically significant ($p > 0.05$); females were unaffected. Bodyweights in animals receiving 15 or 50 mg/kg/day were considered to have been unaffected by treatment. Refer to Table A6_3_1-4 for group mean values.
4.3	**Food consumption and compound intake**	Food consumption was unaffected by treatment. Slightly low food intake was recorded for all treated females. There was, however, no dosage relationship and this was considered to reflect normal biological variation rather than an effect of treatment. Refer to Table A6_3_1-5 for mean group values. Food conversion efficiencies varied considerably but there were no clear treatment-related trends and, consequently, the inter-group differences were not considered to have been due to treatment. Refer to Table A6_3_1-6 for mean group values.
4.4	**Opthalmoscopic examination**	Not performed
4.5	**Blood analysis**	
4.5.1	Haematology	Increased lymphocyte counts, when compared with the controls, were evident on Day 29 in males and females receiving 150 mg/kg/day ($p < 0.01$ and $p < 0.05$ respectively). The neutrophil counts of males receiving 150 mg/kg/day were also raised ($p < 0.01$). These changes resulted in elevated total leucocyte counts in males and females receiving 150 mg/kg/day ($p < 0.001$ and $p < 0.05$ respectively). A few other inter-group differences attained statistical significance when compared with controls ($p < 0.05$) but they were minor or lacked dosage relationship and were considered to represent normal variation. These changes included the low mean cell haemoglobin concentrations in males receiving 150 mg/kg/day which were not attributed to treatment since males receiving 15 mg/kg/day showed a similar change, but those receiving 50 mg/kg/day had values similar to the controls.

		The cellularity and composition of the bone marrow was unaffected by treatment.
4.5.2	Clinical chemistry	On Day 29 of treatment the total protein concentrations of males receiving 50 or 150 mg/kg/day were slightly low ($p < 0.05$ and $p < 0.01$, respectively) in comparison with the controls. Albumin concentrations and albumin to globulin ratios were unaffected, suggesting a reduction in all protein fractions. Slightly low sodium concentrations were evident in males receiving 150 mg/kg/day and high potassium concentrations were evident in females receiving150 mg/kg/day. The inter-group differences were generally small and, as these were confined to one sex they could not, with any confidence, be attributed to treatment. A few other inter-group differences attained statistical significance but were minor or lacked dosage relationship and were considered to represent normal variation.
4.5.3	Urinalysis	Not performed
4.6	**Sacrifice and pathology**	
4.6.1	Organ weights	There were no inter-group differences in organ weights after 28 days of treatment that could, with any confidence, be attributed to treatment.
4.6.2	Gross and histopathology	Macroscopic Pathology Treatment-related changes were confined to the stomach. Thickening of the stomach wall and depressed areas were seen in males and females given 150 mg/kg/day of [redacted] BIT but not in controls or rats that received the lower dosages. Other changes which were observed were those normally encountered in young CD rats at these laboratories. Microscopic Pathology Treatment-related changes were present in the stomach. These consisted of hyperplasia, hyperkeratosis, inflammation and ulceration of the keratinised region and inflammation, mucosal atrophy and mucous cell hypertrophy of the glandular region. These changes were seen in males and females given 150 mg/kg/day, but not in Controls, so microscopic examination was extended to the stomachs of all animals on study. Examination of the stomachs of animals given 15 or 50 mg/kg/day of [redacted] BIT revealed hyperplasia and hyperkeratosis of the keratinised region in some males and females receiving 50 mg/kg/day and slight mucosal atrophy with inflammation in the glandular region of one female receiving 50 mg/kg/day. There were no significant

findings in animals receiving 15 mg/kg/day.

Erythrocytes in the sinuses of the mesenteric lymph nodes, with erythrophagia, were seen in a few males given 150 mg/kg/day. This was not severe in all cases and was possibly secondary to the pathology in the stomach.

All other microscopic findings were considered to be incidental and of no toxicological significance.

The macropathology and histopathology observations are summarised in Tables A6_3_1-7 and A6_3_1-8, respectively.

4.7 Other

None

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and methods

Groups of five male and five female CD rats received [redacted] BIT orally, by gavage, at dosages of 15, 50 or 150 mg/kg/day for four weeks in a study designed to meet the requirements of OECD Guideline 407. The control group received the vehicle (0.5% methylcellulose in purified water) alone. The animals were allowed to acclimatise for seven days prior to the start of the treatment. At the start of the study the animals were 28 to 35 days old and between 96 and 119 g in weight.

The homogeneity and storage stability of [redacted] BIT in 0.5% methyl cellulose was assessed as well as the accuracy of the solutions used for dosing.

Each animal room was supplied with fresh filtered air, with a target temperature of 21 °C, humidity of 55% and a controlled 12 hour light/dark cycle. The animals were fed *ad libitum* with a commercially available pelleted rodent diet and water was freely available.

The food consumption of each group was calculated and from these records the mean weekly consumption per animal was calculated for each cage. The group mean food conversion efficiencies were also calculated. The body weight of each animal was recorded during the acclimatisation period, on the day that treatment commenced, at weekly intervals throughout the treatment period and prior to necropsy.

Group observations were made at least twice daily and individual observations of all animals were made daily during week 1 and twice weekly during weeks 2, 3, and 4 at five intervals throughout the day. Open field observations (for example activity, respiration rate, urination and salivation), sensory reactivity tests (for example auditory pinna reflex, body temperature and pupil closure response behaviour) and grip strength and motor activity tests were also performed before commencement of treatment and during each week of treatment.

During week 4 of treatment blood samples were collected into EDTA

from all animals and all samples were analysed using a Technicon H1 haemotology analyser. Further blood samples were also collected into lithium heparin and for clinical chemistry analysis. Bone marrow samples were obtained from the femur at the necropsy of all animals.

All animals were subjected to a detailed necropsy and organ weights were recorded. Tissue samples from the adrenals, brain, heart, kidneys, liver, lungs, spinal cord, stomach, thyroid and uterus were preserved in formaldehyde, embedded in paraffin wax and 5 µm sections were stained with haematoxylin and eosin.

The significance of inter-group differences in the data collected was assessed using statistical analysis.

5.2 Results and discussion

A female receiving 150 mg/kg/day was killed *in extremis*. Ante mortem respiratory problems indicated that this may have been an accidental death.

Salivation was observed from Day 1 in females and from Day 4 in males receiving 150 mg/kg/day; a few animals receiving 50 mg/kg/day were occasionally affected. Hunched posture was observed in one female and all males receiving 150 mg/kg/day.

The weight gains of males receiving 150 mg/kg/day were slightly low in comparison with the Controls. Food intake and food conversion efficiencies were unaffected by treatment.

Open field observations during Week 2 of treatment indicated moderate or marked salivation in four males and one female receiving 150 mg/kg/day. This was accompanied by chin or muzzle rubbing onto the floor of the open field and unusual 'snaking' body movements. During Weeks 3 and 4 of treatment, one male and one female receiving 150 mg/kg/day were similarly affected.

No treatment-related changes were identified in the sensory reactivity tests or grip strength and motor activity measurements performed in Week 4.

Haematological changes on Day 29 were confined to the high dosage and comprised high lymphocyte counts in males and females and high neutrophil counts in males, the composition of which produced an elevation of total leucocyte count in both sexes.

Bone marrow composition was unaffected by treatment.

Total protein concentrations in males receiving 50 or 150 mg/kg/day were lower than those of the controls on Day 29.

Organ weights were unaffected by treatment.

Macroscopic examination on Day 29 indicated thickening of the stomach wall and depressed areas in males and females given 150 mg/kg/day.

Histopathological changes related to treatment at 150 mg/kg/day were

		confined to the stomach and comprised hyperplasia, hyperkeratosis, inflammation and ulceration of the keratinised region and inflammation, mucosal atrophy and mucous cell hypertrophy of the glandular region. In addition, hyperplasia and hyperkeratosis of the keratinised region was evident in a few males and females given 50 mg/kg/day and slight mucosal atrophy with inflammation was evident in the glandular region of one female given 50 mg/kg/day.	
5.3	**Conclusion**	It is concluded that oral administration of [redacted] BIT at dosages of 50 or 150 mg/kg/day produced changes in the stomach that were consistent with a response to an irritant material and all other findings on this study could be attributed to this response. The no-observed-effect level (NO(A)EL) in this study was 15 mg/kg/day.	
5.3.1	LO(A)EL	50 mg/kg/day	
5.3.2	NO(A)EL	15 mg/kg/day	
5.3.3	Other	None	
5.3.4	Reliability	1	X
5.3.5	Deficiencies	No	

Evaluation by Competent Authorities

EVALUATION BY RAPPORTEUR MEMBER STATE

Date	*July 2021*
Materials and Methods	*Applicant's version is accepted.*
Results and discussion	*Applicant's results and conclusions are accepted.*
Conclusion	*LO(A)EL: 50 mg/kg bw/day* *NO(A)EL: 15 mg/kg bw/day* *Applicants's conclusions are adopted.*
Reliability	*2 (some parameters of the most recent versions of the guide are missing)*
Acceptability	*Acceptable.*

Section A6 Toxicological and Metabolic Studies

Subsection A6.3.1 **REPEATED DOSE TOXICITY**

Annex Point IIA, VI.6.3 **28 Day Rat Oral Gavage**

Remarks

Table A6_3_1-1: Experimental Design

Group	Treatment	Dosage (mg/kg)	Number of Animals		Cage Numbers		Animal Numbers	
			Male	Female	Male	Female	Male	Female
1	Control	N/A	5	5	3	7	11-15	31-35
2	[redacted] BIT	15	5	5	1	8	1-5	36-40
3	[redacted] BIT	50	5	5	4	6	16-20	26-30
4	[redacted] BIT	150	5	5	2	5	6-10	21-25

Table A6_3_1-2: Formulation Analysis – Week 1

Group	Nominal Concentration (mg/mL)	Replicate 1 Determined Concentration (mg/mL)	Replicate 2 Determined Concentration (mg/mL)	Mean Determined Concentration (mg/mL)	% of Nominal Concentration
1	0	ND	ND	ND	-
2	1.5	1.51	1.44	1.48	99
3	5	5.20	5.25	5.23	105
4	15	16.4	16.3	16.4	109

Table A6_3_1-3: Formulation Analysis – Week 4

Group	Nominal Concentration (mg/mL)	Replicate 1 Determined Concentration (mg/mL)	Replicate 2 Determined Concentration (mg/mL)	Mean Determined Concentration (mg/mL)	% of Nominal Concentration
1	0	ND	ND	ND	-
2	1.5	1.49	1.52	1.51	101
3	5	5.01	4.96	4.99	100
4	15	15.2	14.8	15.0	100

Table A6_3_1-4: Bodyweight (Group Mean Values)

Week	Bodweight Data	Group and Sex							
		1M	2M	3M	4M	1F	2F	3F	4F
0	N	5	5	5	5	S	5	5	5
	Mean	105	105	105	104	111	109	110	111
	SD	4.3	5.9	7.6	6.3	6.6	6.0	8.1	4.5
1	N	5	5	5	5	5	5	5	5
	Mean	155	153	149	149	156	147	144	149
	SD	10.7	7.1	9.4	11.7	9.8	5.5	9.6	5.7
2	N	5	5	5	5	5	5	5	4
	Mean	209	206	196	197	184	174	171	179
	SD	17.2	8.7	15.0	20.9	15.7	10.3	8.7	9.2
3	N	5	5	5	5	5	5	5	4
	Mean	260	253	244	228	208	193	189	199
	SD	20.0	10.4	21.6	48.9	18.0	14.8	8.9	7.2
4	N	5	5	5	5	5	5	5	5
	Mean	280	271	262	248	209	196	195	202
	SD	23.7	15.5	21.9	57.3	19.2	15.3	10.0	8.4
Gain		176	165	157	144	98	87	84	90
As % of Control		N/A	94	89	82	N/A	89	86	92

Groups 1, 2, 3, and 4 are Control and 15, 50 and 150 mg/kg/day dose groups, respectively
M = Male, F= Female
N = Number of animals
SD = Standard Deviation
N/A = Not applicable

Table A6_3_1-5: Food Consumption – Group Mean Values (g/rat)

Week Number	Group and Sex							
	1M	2M	3M	4M	1F	2F	3F	4F
1	150	146	*	153	155	139	136	142
2	178	175	172	172	147	131	133	139
3	186	184	182	163	145	126	132	143
4	131	192	176	161	136	126	140	114
Total	645	697	**	649	583	522	541	538
As % of Control	N/A	108	N/A	101	N/A	90	93	92

Groups 1, 2, 3, and 4 are Control and 0, 15, 50 and 150 mg/kg/day dose groups, respectively
M = Male, F= Female
* = Value excluded: low re-fill weight recorded
** = Not calculated since week 1 data was excluded
N/A = Not applicable

Table A6_3_1-6: Food Conversion Efficiency- Group Mean Values Expressed as a Percentage

Week Number	Group and Sex							
	1M	2M	3M	4M	1F	2F	3F	4F
1	33.1	32.3	*	28.9	28.7	27.1	24.7	26.9
2	30.6	30.5	27.3	28.0	19.3	20.6	20.1	15.7
3	27.5	25.6	26.4	19.4	16.6	14.8	13.9	14.2
4	31.8	20.4	23.0	29.3	15.5	12.0	15.7	17.8
Mean	27.3	23.7	**	22.2	16.8	16.7	15.5	16.7

Groups 1, 2, 3, and 4 are Control and 0, 15, 50 and 150 mg/kg/day dose groups, respectively
M = Male, F= Female
* = Value excluded since there was no food consumption value
** = Not calculated since week 1 data was excluded
N/A = Not applicable

Table A6_3_1-7: Macropathology – Group Distribution of Findings for Animals Killed After 28 Days of Treatment

Sex		**Male**				**Female**			
Group		1	2	3	4	1	2	3	4
Number of Animals in Group		5	5	5	5	5	5	5	4
Organ		**Number of Animals Examined / Observation**							
Liver × 2	Examined	5	5	5	5	5	5	5	4
	Area(s)of Change	0	1	0	0	0	0	0	0
Thymus	Examined	5	5	5	5	5	5	5	4
	Finding	1	0	0	0	0	0	0	0
Lymph Node (Mesentric)	Examined	5	5	5	5	5	5	5	4
	Dark	0	0	0	1	0	0	0	0
Kidneys	Examined	5	5	5	5	5	5	5	4
	Hydronephorsis	0	0	0	0	0	0	1	0
Uterus	Examined	0	0	0	0	5	5	5	4
	Fluid Distention	0	0	0	0	2	1	3	1
Stomach × 2	Examined	5	5	5	5	5	5	5	4
	Thickened Wall	0	0	0	2	0	0	0	1
	Depressed Area(s)	0	0	0	3	0	0	0	3*
Miscellaneous	Examined	5	5	5	5	5	5	5	4
	Thin	0	0	0	1	1	0	0	1

Group 1: Control;
Group 2: 15 mg/kg [redacted] BIT
Group 3: 50 mg/kg [redacted] BIT
Group 4: 150 mg/kg [redacted] BIT
* Significant when compared to Group 1 ($P < 0.05$)

Table A6_3_1-8: Histopathology – Group Distribution of Findings for Animals Killed After 28 Days of Treatment

Sex		**Male**				**Female**			
Group		1	2	3	4	1	2	3	4
Number of Animals in Group		5	5	5	5	5	5	5	4
Organ		**Number of Animals Examined / Observation**							
Heart, Ventricle	Examined	5	0	0	5	5	0	0	4
	Myocarditis	0	0	0	1	3	0	0	0
Kidneys	Examined	5	5	5	5	5	5	5	4
	Basophilic Cortical Tubules	1	1	1	2	1	1	1	0
	Hydronephrosis	0	0	0	2	0	0	1	0
	Interstitial Inflammation	1	1	1	0	0	1	0	0
	Medullary Cyst(s)	0	0	1	0	0	0	0	0
	Cortico-Medullary Mineralisation	0	0	0	0	3	3	4	2
	Dilated Papillary Collecting Duct	0	0	0	0	1	0	0	0
Lymph Node (Mandibular)	Examined	5	0	0	5	5	0	0	4
	Plasmacytosis	0	0	0	1	1	0	0	1
	Erythrocytes and Erythrophagocytosis in Sinuses	0	0	0	2	0	0	0	0
Lymph Node (Mesentric)	Examined	5	0	0	5	5	0	0	4
	Erythrocytes and Erythrophagocytosis in Sinuses	0	0	0	3	0	0	0	0
Liver x 2	Examined	5	5	5	5	5	5	5	4
	Chronic Inflammatory Cell Foci	1	1	2	2	2	3	2	2
Lungs x 2	Examined	5	5	5	5	5	5	5	4

	Alveolar Haemorrhage	0	1	1	0	2	0	1	1
	Focal Pneumonia	0	0	1	0	1	0	0	0
	Accumulation(s) of Alveolar Macrophages	0	0	0	0	0	0	0	1
Prostate	Examined	5	0	0	5	0	0	0	0
	Chronic Inflammation	0	0	0	1	0	0	0	0

Group 1: Control;
Group 2: 15 mg/kg [redacted] BIT
Group 3: 50 mg/kg [redacted] BIT
Group 4: 150 mg/kg [redacted] BIT
* Significant when compared to Group 1 ($P < 0.05$)

Table A6_3_1-8 (Continued): Histopathology – Group Distribution of Findings for Animals Killed After 28 Days of Treatment

Sex		**Male**				**Female**			
Group		1	2	3	4	1	2	3	4
Number of Animals in Group		5	5	5	5	5	5	5	4
Organ		**Number of Animals Examined / Observation**							
Stomach × 2	Examined	5	5	5	5	5	5	5	4
	Submucosal Oedema	0	0	0	1	0	0	0	0
	Keratinised Region: Acute Inflammation	0	0	0	1	0	0	0	0
	Keratinised Region: Ulcers(s)	0	0	0	1	0	0	0	1
	Keratinised Region: Hyperkeratosis	0	0	3	4*	0	0	2	3*
	Keratinised Region: Epithelial Hyperplasia	0	0	3	5**	0	0	1	4**
	Keratinised Region: Chronic Inflammation	0	0	0	3	0	0	0	3*
	Keratinised Region: Parakeratosis	0	0	0	1	0	0	1	0
	Glandular Region: Mucosal Atrophy	0	0	0	4*	0	0	1	0
	Glandular Region: Submucosal Inflammation	0	0	0	1	0	0	0	1
	Glandular Region: Mucosal Inflammation	0	0	0	2	0	0	1	0
	Glandular Region: Mucous Cell Hypertrophy	0	0	0	4*	0	0	0	1
Thyroids)	Examined	5	0	0	5	5	0	0	4
	Ectopic Thymic Tissue	3	0	0	0	3	0	0	1
	Chronic Inflammation	0	0	0	0	0	0	0	1
Uterus	Examined	0	0	0	0	5	1	3	4
	Dilated	0	0	0	0	2	1	3	1

Group 1: Control;
Group 2: 15 mg/kg [redacted] BIT
Group 3: 50 mg/kg [redacted] BIT
Group 4: 150 mg/kg [redacted] BIT
* Significant when compared to Group 1 (P < 0.05)
** Significant when compared to Group 1 (P < 0.01)

Table A6_3_1-9: Summary of Results of Repeat Dose Toxicity to [redacted] BIT

Parameter	Control		15 mg/kg		50 mg/kg		150 mg/kg	
	Male	Female	Male	Female	Male	Female	Male	Female
number of animals examined	5	5	5	5	5	5	5	5
number of mortalities	-	-	-	-	-	-	-	11
clinical signs					↑	↑	↑	↑
body weight	-	-	-	-	-	-	↓	-
food consumption	-	-	-	-	-	-	-	-
clinical chemistry	-	-	-	-	↓↓	-	↓↓	-
haematology							↑↑	↑↑
organ weight	-	-	-	-	-	-	-	-
gross pathology	-	-	-	-	-	-	3	3
microscopic pathology	-	-	-	-	4	4	5	5

[1] Mortality considered to be unrelated to treatment
↑: Salivation immediately after dosing was observed on most days during the study in animals receiving 150 mg/kg/day. Occasionally, in a few animals the salivation was seen before dosing. In addition, a few animals receiving 50 mg/kg/day occasionally salivated immediately after dosing.
↓: Males receiving 150 mg/kg/day gained slightly less weight than Controls. This difference was, however, not statistically significant ($p > 0.05$); females were unaffected.
↓↓: Total protein concentrations were observed to be slightly low. $P < 0.05$ and $P < 0.01$ for 50 mg/kg/day and 150 mg/kg/day, respectively.
↑↑: Elevated leucocyte counts in males and females ($p < 0.001$ and $p < 0.05$, respectively).
3: Treatment-related changes were confined to the stomach. Thickening of the stomach wall and depressed areas were observed in males and females.
4: Treatment related changes were observed in the stomach, consisting of hyperplasia and hyperkeratosis of the keratinised region in some males and females receiving 50 mg/kg/day and slight mucosal atrophy with inflammation in the glandular region of one female receiving 50 mg/kg/day.
5: Treatment related changes were observed in the stomach, consisting of hyperplasia, hyperkeratosis, inflammation and ulceration of the keratinised region and inflammation, mucosal atrophy and mucous cell hypertrophy of the glandular region. These changes were seen in males and females given 150 mg/kg/day.

Section A6	**Toxicological and Metabolic Studies**	
Subsection A6.3.2	**Short-term repeated-dose toxicity test**	
Annex Point IIA VI.6.3.2	**SHORT-TERM REPEATED DOSE DERMAL TOXICITY**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ]	**Technically not feasible [X]** **Scientifically unjustified [X]**	
Limited exposure [X]	**Other justification [ ]**	
Detailed justification:	According to the TNsG on data requirements, repeat toxicity tests are used as a range-finding tests and are not required when an adequate sub-chronic toxicity study is available in a rodent. The Repeat Dermal study is also usually required when the dermal route of exposure is significant and the compound is known to be toxic by the dermal route and can penetrate through intact skin. This study is not required on the following basis; • An adequate sub-chronic toxicity study is available in a rodent (See Section 6.4.1). • Acute dermal toxicity studies showed only minor toxic effects at the highest dose tested (See Section 6.1.2). • It is also possible to calculate the route-to-route exposure from available oral toxicity studies and using dermal penetration studies (Section 6.2) as there are no specific effects observed following dermal exposure in animals. Therefore an accurate and realistic determination of dermal toxicity can be derived from available sub-chronic oral exposure studies and *in vitro* dermal penetration studies.	
Undertaking of intended data submission [ ]		
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*September 2008*	
Evaluation of applicant's justification	*Applicant's justification is acceptable.*	
Conclusion	*Applicant is exempted of the dermal repeated dose toxicity study.*	
Remarks		

Section A6	**Toxicological and Metabolic Studies**	
Subsection A6.3.3	**Short-term repeated-dose toxicity test**	
Annex Point IIA VI.6.3.3	**SHORT-TERM REPEATED DOSE INHALATION TOXICITY**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ]	**Technically not feasible [X]** **Scientifically unjustified [X]**	
Limited exposure [X]	**Other justification [ ]**	
Detailed justification:	According to the TNsG on data requirements, repeat toxicity tests are used as a range-finding tests and are not required when an adequate sub-chronic toxicity study is available in a rodent. The Repeat Inhalation study is also required for volatile substances (vapour pressure > 1 x 10^{-2} Pa) or in cases where the potential inhalation exposure is significant. This study is not required on the following basis; • An adequate sub-chronic toxicity study is available in a rodent (See Section 6.4.1). • Based on the intrinsic properties of the test substance (See Justification for non-submission of data for Section 6.1.3), inhalation exposure is not expected, and technically unfeasible to simulate in laboratory tests. Therefore non-inclusion of this data requirement is justified on the basis that the criteria for requirement are not met.	
Undertaking of intended data submission [ ]		
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*September 2008*	
Evaluation of applicant's justification	*Applicant's justification is acceptable.*	
Conclusion	*Applicant is exempted of the dermal repeated dose toxicity study.*	
Remarks		

Section A6 Toxicological and Metabolic Studies

Subsection A6.4.1/1 Repeated dose toxicity

Annex Point IIA VI.6.4.1.a/01 **SUBCHRONIC ORAL TOXICITY TEST IN RATS (90 DAYS)**

			Official use only
		1 REFERENCE	
1.1	**Reference**	[redacted] 1991; [redacted] 90 Day Feeding Study in Rats. [redacted] Report No. [redacted]P/3183	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Arch Chemicals Inc	
1.2.2	Company with letter of access	Clariant Production UK Ltd and Thor GmbH.	
1.2.3	Criteria for data protection	Data on existing substance for first entry into Annex I.	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	US EPA PAG 82-1	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	[redacted]	
3.1.2	Specification	Deviating from specification given in section 2 as follows: The Test Substance employed was pre-dried technical grade active substance.	
3.1.2.1	Purity	93.3%	
3.1.2.2	Stability	The purity used for purposes of diet preparation (treatment commenced between 19 and 21 June 1990) was 93.3%. The test item was stored at ambient temperature and was certified with a purity of 93.3% on 20 May 1991. The experimental diets were prepared in 30 kg batches from premixes prepared by triturating the appropriate amount of [redacted] with 500 g of milled diet. The premixes were then added to 29.5 kg of diet and mixed thoroughly. The powdered diets were dispensed into jars and stored at -20 °C. One jar at each dose level for each cage was removed every third day from the freezer, allowed to defrost for three hours and then presented	

		to the animals, ensuring that the experimental diets available to the animals were changed every third day.
3.2	**Test Animals**	
3.2.1	Species	Rat
3.2.2	Strain	Alpk:APfSD (Wistar derived)
3.2.3	Source	[redacted] All rats were supplied as weanlings (22-23 days old) segregated by sex and litter of origin. They were transported to the testing laboratory in containers which were sealed within a polythene sleeve at the breeding unit. This sleeve contained sufficient air for the journey. The sealed containers were introduced to the barriered Unit via a dunk tank and the sleeve was then removed. This procedure ensured that the Specific Pathogen Free status of the rats was maintained during transfer from the Breeding Unit. For 10 days following delivery of the rats, personnel access to the animal room was restricted as a quarantine procedure.
3.2.4	Sex	Male and female
3.2.5	Age/weight at study initiation	6-7 weeks old/males 152 to 230 g, females 133 to 170 g
3.2.6	Number of animals per group	24 Refer to Table A6_4_1(1)-1 for details of the experimental design. During an acclimatisation period of approximately 3 weeks, the rats were randomly allocated to cages using a procedure which ensured that each litter was equally represented in all dose groups (including controls). At randomisation, each rat was uniquely identified by ear punching with the number assigned to it by the experimental design. During acclimatisation and dosing the animal room was generally maintained at 19-22°C and a minimum nominal relative humidity of 40%. Twelve hour periods of light were alternated with twelve hour periods of darkness. The ventilation system was designed to provide a minimum of fifteen air changes per hour. The room in which the study was accommodated contained animals from this study only. The rats were housed, sexes separately, in multiple rat racks in groups of four.
3.2.7	Control animals	Yes

Section A6 Toxicological and Metabolic Studies

Subsection A6.4.1/1 Repeated dose toxicity

Annex Point IIA VI.6.4.1.a/01 **SUBCHRONIC ORAL TOXICITY TEST IN RATS (90 DAYS)**

3.3	**Administration/ Exposure**	Oral
3.3.1	Duration of treatment	90 days
3.3.2	Frequency of exposure	Daily
3.3.3	Post-exposure period	Not applicable
3.3.4	**Oral**	
3.3.4.1	Type	Dietary
3.3.4.2	Concentration	0, 200, 900 and 4000 ppm The mean dose received (mg/kg/day) over the 90 day period is detailed below. The weekly averages are presented in Table A6_4_1(1)-2.

Dietary Concentration of [redacted]					
200 ppm		900 ppm		4000 ppm	
Mean Dose Received Over 13 Weeks of Treatment (mg/kg/day)					
Male	Female	Male	Female	Male	Female
15.3	17.6	69.0	78.3	322.0	356.3

The dose levels for this study were selected on the basis of results from a 28 day feeding study in the Alpk:APfSD rat, previously performed by the testing laboratory.

3.3.4.3	Controls	CT1 diet supplied by Special Diets Services Limited, Stepfield, Witham, Essex, UK.

3.3.4.4	Concentration in vehicle	0, 200, 900 and 4000 ppm The homogeneity of [redacted] in CT1 diet was determined by analysing samples from all dose levels. The storage stability of [redacted] in diet was determined for all concentrations over a period of 40 days at -20 °C. Analysis was performed after storage at ambient temperature over a 40-day period for the 4000 ppm diet and over a 4- and 7-day period for the 900 ppm diet. Ambient storage stability was initially determined after 4 and 6 days for the 200 ppm diet, however there was a reduction in the percentage recovery of [redacted] at this dietary level after 4 days storage. Therefore the analysis was repeated after 1 and 2 days storage. Samples from all dietary levels (including the control group) were taken at intervals throughout the study and analysed quantitatively for [redacted] All test diet samples were analysed in duplicate together with procedural recovery samples which were analysed in triplicate. The diet samples were Soxhlet extracted with acetonitrile and the final extracts analysed together with a calibration line (the calibration standards were prepared using [redacted] by high performance liquid chromatography using a Spherisorb S5ODS2 column and UV detection at 250 nm. The limit of detection of the analytical method was 8 ppm and the samples were quantified using a linear regression progrmme or by direct comparison of the detector response to a calibration standard.
3.3.4.5	Total volume applied	Refer to Tables A6_4_1(1)-5 and A6_4_1(1)-6 for an inter-group comparison of male and female food consumption.
3.3.4.6	Controls	Diet controls
3.4	**Examinations**	
3.4.1	Observations	
3.1.2.3	Clinical signs	Prior to the start of the study all rats were examined to ensure that they were physically normal and they exhibited normal activity. During the study all rats were observed daily for changes in clinical condition and behaviour and once weekly a detailed examination of each rat was made. Any abnormalities together with the observation of no abnormality detected were recorded.
3.1.2.4	Mortality	Observations were made daily. Any rats requiring euthanasia were killed and subjected to a post mortem examination. Any rats found dead were subjected to a post

		mortem examination as soon as possible after death.
3.4.2	Body weight	The body weight of each rat was recorded in replicate order immediately before feeding the experimental diet commenced and then on the same day of each subsequent week until termination. Initial bodyweights (Day 1) were considered by analysis of variance and bodyweights for all subsequent weeks by analysis of covariance on initial bodyweight, separately for males and females. The differences from control based on the analysis of bodyweight adjusted for initial weight and food consumption were also analysed graphically. A graph was plotted for each dose group for males and females. A bar was used to represent the mean percentage difference between control and treated group least square means, the top and bottom of each bar represents the upper and lower 95% confidence limits for this difference. If the bar does not cross the zero difference line at a particular week, there is a statistically significant difference between the treated group and the control at that week. Analysis of variance and covariance allowed for the replicate structure of the study design and also for litter of origin. All analyses were carried out using the GLM procedure in SAS (1985). Least square means for each group were calculated using the LSMEAN option in SAS PROC GLM. Unbiased estimates of differences from control were provided by the difference between each treatment group least square mean and the control group least square mean. Differences from control were tested statistically by comparing each treatment group least square mean with the control group least square mean using a two-sided Student's t-test, based on the error mean square in the analysis. All data were checked for unusual values and where such values were detected the analyses were repeated omitting these values to determine their influence on the conclusions.
3.4.3	Food consumption	Food consumption for each cage of rats was recorded continuously throughout the study and calculated on a weekly basis. The food utilisation value per cage was calculated as the bodyweight gained by the rats in the cage per 100 g food eaten. Weekly food consumption, total food consumption and food utilisation during the of period 1-4, 5-8, 9-13 and 1-13 weeks were considered by analysis of variance, separately for males and females. Food utilisation was calculated as: ((bodyweight gain (g) per cage over time period)/ (food consumed per cage over time period)) × 100 Analysis of variance allowed for the replicate structure of the study design. All analyses were carried out using the GLM procedure in SAS (1985). Least square means for each group were calculated using

		the LSMEAN option in SAS PROC GLM. Unbiased estimates of differences from control were provided by the difference between each treatment group least square mean and the control group least square mean. Differences from control were tested statistically by comparing each treatment group least square mean with the control group least square mean using a two-sided Student's t-test, based on the error mean square in the analysis. All data were checked for unusual values and where such values were detected the analyses were repeated omitting these values to determine their influence on the conclusions.
3.4.4	Water consumption	Not recorded
3.4.5	Ophthalmoscopic examination	The eyes of all animals given 0 or 4000 ppm were examined pre-experimentally and during the week prior to termination. The examination was carried out using a Fison's binocular indirect ophthalmoscope. Animals were examined after instillation of 0.5% v/v tropicamide into the eyes to dilate the pupils.
3.4.6	Haematology	At termination, all rats were bled by cardiac puncture and samples collected in tubes containing EDTA as an anticoagulant. The following parameters were determined: haemoglobin, total white cell count, red cell count, mean cell volume, mean cell haemoglobin, mean cell haemoglobin concentration, haematocrit and platelet count. These values were measured using an ELT-800 (*Ortho* Diagnostics PLC). Further samples of blood were collected in tubes containing 0.11 M trisodium citrate as an anticoagulant and prothrombin and kaolin-cephalin times were measured on a 'Coag-a-mate' (Organon Teknika). In addition, a differential white cell count was performed on samples from the control and 4000 ppm groups on a Romanowsky-stained blood film and the morphological appearance of red cells was examined. Haematology was considered by analysis of variance. Male and female data were analysed and the results examined to determine whether any differences between control and treated groups were consistent between sexes. Analysis of variance allowed for the replicate structure of the study design and also for litter of origin. All analyses were carried out using the GLM procedure in SAS (1985). Least square means for each group were calculated using the LSMEAN option in SAS PROC GLM. Unbiased estimates of differences from control were provided by the difference between each treatment group least square mean and the control group least square mean. Differences from control were tested statistically by comparing each treatment group least square mean with the control group least square mean using a two-sided Student's t-test, based on the error mean square in the analysis.

		All data were checked for unusual values and where such values were detected the analyses were repeated omitting these values to determine their influence on the conclusions.
3.4.7	Clinical chemistry	Blood samples were also collected at termination in tubes containing lithium heparin as an anticoagulant. Samples were measured for the following parameters in plasma: urea, creatinine, glucose, albumin, total protein, cholesterol, triglycerides, bilirubin, chloride, sodium, potassium, calcium and phosphorus (as phosphate) and alkaline phosphatase, alanine transaminase, creatine kinase, aspartate transaminase and *gamma*-glutamyl transferase activities. All these parameters were measured on a Kone Specific analyser. Clinical biochemistry was considered by analysis of variance. Male and female data were analysed and the results examined to determine whether any differences between control and treated groups were consistent between sexes. Analysis of variance allowed for the replicate structure of the study design and also for litter of origin. All analyses were carried out using the GLM procedure in SAS (1985). Least square means for each group were calculated using the LSMEAN option in SAS PROC GLM. Unbiased estimates of differences from control were provided by the difference between each treatment group least square mean and the control group least square mean. Differences from control were tested statistically by comparing each treatment group least square mean with the control group least square mean using a two-sided Student's t-test, based on the error mean square in the analysis. All data were checked for unusual values and where such values were detected the analyses were repeated omitting these values to determine their influence on the conclusions.
3.4.8	Urinalysis	Not performed
3.5	**Sacrifice and pathology**	
3.5.1	Organ weights	Testes, adrenal glands, kidneys, liver and brain were weighed. Paired organs were weighed together.

Section A6 Toxicological and Metabolic Studies

Subsection A6.4.1/1 Repeated dose toxicity

Annex Point IIA VI.6.4.1.a/01 **SUBCHRONIC ORAL TOXICITY TEST IN RATS (90 DAYS)**

3.5.2	Gross and histopathology	Macropathology A full macroscopic examination was performed on all animals. The following tissues were excised: Adrenal gland, aorta, bone (femur including knee), bone marrow (femur), brain, caecum, cervix, colon, duodenum, epididymis, eye, Harderian gland, heart, ileum, jejunum, kidney, liver, lung, lymph node-cervical, lymph node-mesenteric, mammary gland (inguinal), nasal cavity, oesophagus, oral cavity, ovary, pancreas, parathyroid gland, pituitary gland, prostate gland, rectum, salivary gland, sciatic nerve, seminal vesicle, skin, spinal cord, spleen, sternum, stomach, testis, thymus, thyroid gland, trachea, urinary bladder, uterus, voluntary muscle and any abnormal tissue. Histopathology The issues detailed above were fixed in 10% neutral buffered formol saline with the exception of the testis, epididymis, skin and mammary gland which were fixed in Bouin's fluid, and the eye and Harderian gland which were fixed in Davidson's solution. The nasal cavities of all rats were perfused with 10% neutral buffered formol saline. Tissues from all groups, except the sternum and oral and nasal cavity which were stored, were routinely embedded in paraffin wax. 5 µm sections of processed liver, kidney, lung and stomach from all treatment groups (including the control group) and all processed tissues from the control and 4000 ppm groups were cut and then stained with haematoxylin and eosin for examination by light microscopy.
3.5.3	Other examinations	Not applicable
3.5.4	Statistics	Organ weights were considered by analysis of variance and analysis of covariance on final bodyweight, separately for males and females. Analysis of variance and covariance allowed for the replicate structure of the study design and also for litter of origin. All analyses were carried out using the GLM procedure in SAS (1985). Least square means for each group were calculated using the LSMEAN option in SAS PROC GLM. Unbiased estimates of differences from control were provided by the difference between each treatment group least square mean and the control group least square mean. Differences from control were tested statistically by comparing each treatment group least square mean with the control group least square mean using a two-sided Student's t-test, based on the error mean square in the analysis. All data were checked for unusual values and where such values were detected the analyses were repeated omitting these values to determine their influence on the conclusions.

Section A6 Toxicological and Metabolic Studies

Subsection A6.4.1/1 Repeated dose toxicity

Annex Point IIA VI.6.4.1.a/01 **SUBCHRONIC ORAL TOXICITY TEST IN RATS (90 DAYS)**

3.6 Further remarks None

4 RESULTS AND DISCUSSION

4.1 Observations

4.1.1 Clinical signs

All animals were observed to be in good clinical condition.

The few findings recorded were of a type and incidence commonly seen in rats of this age and strain and none were considered to be compound related.

4.1.2 Mortality

There were no mortalities.

4.2 Body weight gain

The bodyweights of animals given 4000 ppm were statistically significantly reduced throughout the study, such that the final bodyweights of males and females given 4000 ppm were 6% lower than their respective control values after adjustment for initial bodyweight (this is a statistically significant difference from the control group mean at the 1% level, Student's t-test, two-sided). There was evidence of a marginal difference in the growth of males given 900 ppm, such that the final bodyweights after adjustment for initial weight were 3% lower than the control value. This difference was not statistically significant and considered to be too small to be toxicologically significant. There was no effect on bodyweight at any other dose level.

Refer to Tables A6_4_1(1)-3 and A6_4_1(1)-4 for an intergroup comparison of male and female body weights.

4.3 Food consumption and compound intake

There was some evidence for a reduction in food consumption in all male treatment groups. However, the differences from the control values in all male groups were small and were neither consistent nor dose-related; these differences were considered therefore not to be directly compound-related.

There was also evidence of reduction in food consumption in the 4000 ppm female dose group during weeks 4, 5, 8, 9 and 10. This difference was statistically significant at the 1% level when compared to the control group mean (Student's t-test, two-sided).

The overall reduction in food utilisation apparent in males (statistically significant at the 1% level when compared to the control group mean) and to lesser extent females (not statistically significant) given 4000 ppm was due to the reduced food utilisation apparent during the first 4 weeks of the study.

Refer to Tables A6_4_1(1)-5and A6_4_1(1)-6 for an inter-group comparison of male and female food consumption.

Refer to Tables A6_4_1(1)-7 and A6_4_1(1)-8 for an inter-group

		comparison of male and female food utilisation.
4.4	**Ophtalmoscopic examination**	The eyes of most rats examined were normal and the small numbers of abnormalities seen were similar to those commonly found in rats of this age and strain. None were considered treatment-related.
4.5	**Blood analysis**	
4.5.1	Haematology	Some statistically significant differences between control and [redacted] treated groups were observed but these were small and/or not dose related and were considered not to be treatment-related.
4.5.2	Clinical chemistry	There was a statistically significant increase in plasma alkaline phosphatase activity of males given 4000 ppm and statistically significant increases in plasma cholesterol levels of females given 900 and 4000 ppm. However, these changes were small and considered to be of no toxicological significance. There were other statistically significant differences between control and [redacted] treated groups but these were small and/or not dose-related and therefore were considered not to be treatment-related.
4.5.3	Urinalysis	Not performed
4.6	**Sacrifice and pathology**	
4.6.1	Organ weights	Some statistically significant differences were seen but were considered marginal and/or not dose-related. Refer to Tables A6_4_1(1)-9 to A6_4_1(1)-13 for male and female intergroup comparison of organ weights (adrenals, brain, kidneys, liver and testes).

Section A6 Toxicological and Metabolic Studies

Subsection A6.4.1/1 Repeated dose toxicity

Annex Point IIA VI.6.4.1.a/01 **SUBCHRONIC ORAL TOXICITY TEST IN RATS (90 DAYS)**

4.6.2 Gross and histopathology

The only macroscopic finding attributed to treatment with [redacted] was a thickened limiting ridge in the stomach which was present in ten males and ten females given 4000 ppm and one male and one female given 900 ppm.

Histopathological examination of all tissues (apart from the stomach, lung, kidney and liver which were examined from all animals) was confined to the control and the 4000 ppm groups. In eleven males and eleven females given 4000 ppm there was minimal, slight or moderate hyperplasia of the forestomach adjacent to the limiting ridge. A mixed inflammatory cell submucosal infiltrate accompanied the hyperplastic change in two males and six females. One of the females had minimal ulceration with submucosal haemorrhage in the forestomach and there was slight focal hyperplasia in the oeosophagus of one male given 4000 ppm.

Extra sections of stomach from the block and wet tissue were examined from the male and female given 900 ppm reported as having thickened limiting ridges in the stomach at macroscopic examination. These were normal on microscopic examination.

There were no other microscopic findings considered to be related to treatment with [redacted]

4.7 Other

Dietary Concentration Determined by HPLC Analysis with UV Detection

The concentration of [redacted] in diet prepared at 200, 900 and 4000 ppm on 14 June 1990, 15 July 1990 and 19 August 1990 was within the range 93.5 to 104.2%, 91.0 to 109.2% and 97.7 to 103.5% of the nominal concentration, respectively.

Homogeneity of Diet Determined by HPLC Analysis with UV Detection

The mean concentration of aliquots of diet samples analysed from diet prepared on 14 June at 1990 at 200, 900 and 4000 ppm were observed to be within 10% difference from the overall mean concentration.

Ambient Storage Stability of Diet Determined by HPLC Analysis with UV Analysis

Diet samples at 4000 ppm [redacted] stored for 40 days at ambient temperature were observed to be an average of 91.3% of the initial concentration. Diet samples at 900 ppm stored for 7 days at ambient temperature were observed to be an average of 102.7% of the initial concentration. Diet samples at 200 ppm [redacted] stored for 4 and 6 days at ambient temperature were observed to be an average of 81.3% and 78.6% of the initial concentration, respectively. Since the percentage recovery obtained for the 200 ppm dose level was low after ambient storage for 4 days, additional analysis was performed. After 1 and 2 days ambient storage, the 200 ppm diet samples were observed to be an average of 115.5 and 105.3% of the

initial concentration, respectively.

Frozen Storage Stability of Diet Determined by HPLC Analysis with UV Analysis

Diet samples at 200, 900 and 4000 ppm [redacted] stored for 40 days at -20 °C were observed to be an average of 121.9, 107.8 and 96.3% of the initial concentration, respectively.

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and methods

Groups of twelve male and twelve female Alpk:APfSD rats were fed diets containing 0, 200, 900 or 4000 ppm [redacted] for a period of 90 days. At the start of study the animals were six to seven weeks old, the males were in the range 152 to 230g and the females weighed 133 to 170g. During the acclimatisation period (three weeks), the rats were randomly allocated into groups of four (sexes housed separately), using a procedure which ensured that all animals were equally represented in each groups. The Food consumption and bodyweight were monitored throughout the study and the average dose received by the animals in the 200, 900 and 4000 ppm groups was calculated as 15.3, 69.0 and 322.0 mg/kg/day for males, and 17.6, 78.3 and 356.3 mg/kg/day for females, respectively. The animals were examined prior to the start of the study to ensure that they were physically normal and exhibited normal activity. During the study the rats were observed daily for changes in clinical conditions and behaviour and once weekly a detailed examination of each rat was performed. An ophthalmoscopic examination of all animals in the control and 4000 ppm was performed prior to the start of the study and then during the week prior to termination. After 90 days of treatment all surviving animals were sacrificed and blood samples taken for haematological and clinical biochemistry examination. A full macroscopic examination was performed on all animals and the adrenal glands, kidneys, liver, brain and testes were weighed. Microscopic examination was performed on liver, kidney, lung and stomach samples from all animals and on all processed tissues from the control and 4000 ppm dose group.

5.2 Results and discussion

There were no mortalities, all animals were observed to be in good clinical condition and any clinical signs recorded were considered to be of a type and incidence commonly seen in rats of this age and strain and therefore not considered to be compound related.

The bodyweights of animals given 4000 ppm were statistically significantly reduced throughout the study, such that the final bodyweights of males and females given 4000 ppm were 6% lower than their respective control values after adjustment for initial bodyweight (this is a statistically significant difference from the control group mean at the 1% level, Student’s t-test, two-sided). There was evidence of a marginal difference in the growth of males given 900ppm, such that the final bodyweights after adjustment for

initial weight were 3% lower than the control value. This difference was not statistically significant and considered to be too small to be toxicologically significant. There was no effect on bodyweight at any other dose level.

There was some evidence for a reduction in food consumption in females at 4000 ppm and in all male treatment groups. However, the differences from the control values in all male groups were small and were neither consistent nor dose-related; these differences were therefore considered not to be directly compound-related. There was an overall reduction in food utilisation apparent in males and females (not statistically significant in females) from the 4000 ppm dose group; this was considered to be a result of the reduced food utilisation observed during the first four weeks of dosing.

The eyes of most rats examined were normal and the small numbers of abnormalities seen were similar to those commonly found in rats of this age and strain. None were considered treatment-related.

There were no observations in the haematology determinations considered to be treatment related. In the clinical chemistry determinations, there was a statistically significant increase in plasma alkaline phosphatase activity of males given 4000 ppm and statistically significant increases in plasma cholesterol levels of females given 900 and 4000ppm. There were other statistically significant differences between control and [redacted] treated groups in the clinical chemistry determinations however these were small and/or not dose-related and therefore were not considered to be treatment-related.

Some statistically significant differences were seen in organ weights but these were marginal and/or not dose-related and are considered to be incidental to treatment with [redacted]. The only macroscopic finding attributed to treatment with [redacted] was a thickened limiting ridge in the stomach which was present in ten males and ten females given 4000 ppm and one male and one female given 900ppm. Histopathological examination of all tissues (apart from the stomach, lung, kidney and liver which were examined from all animals) was confined to the control and the 4000 ppm groups. In eleven males and eleven females given 4000ppm there was minimal, slight or moderate hyperplasia of the forestomach adjacent to the limiting ridge. A mixed inflammatory cell submucosal infiltrate accompanied the hyperplastic change in two males and six females. One of the females had minimal ulceration with submucosal haemorrhage in the forestomach and there was slight focal hyperplasia in the oeosophagus of one male given 4000 ppm. Extra sections of stomach from the block and wet tissue were examined from the male and female given 900 ppm reported as having thickened limiting ridges in the stomach at macroscopic examination. These were normal on microscopic examination. There were no other microscopic findings considered to be related to treatment with [redacted]

Section A6 Toxicological and Metabolic Studies

Subsection A6.4.1/1 Repeated dose toxicity

Annex Point IIA VI.6.4.1.a/01 **SUBCHRONIC ORAL TOXICITY TEST IN RATS (90 DAYS)**

		[redacted]	
5.3	**Conclusion**	The administration of 4000 ppm [redacted] for 90 consecutive days resulted in a reduced growth rate without signs of overt toxicity. Histopathological changes in the stomach at 4000ppm were ascribed to an irritant effect of the test substance. The toxicological no effect level, on the basis of this 90 day feeding study, is 900ppm [redacted] The study was conducted in compliance with US EPA PAG 82-1, but can also be considered to be compatible with EC B.26 (a minor exception is that the weights of the epididymides, uterus, ovaries, thymus and heart were not recorded, however, macro- and micro-scopic examinations of these tissues allows their exclusion as target organs for [redacted] toxicity).	
5.3.1	LO(A)EL	4000 ppm equivalent to 322.0 mg/kg/day in males and 356.3 mg/kg/day in females (based on reduced growth rate and histopathological changes in the stomach)	
5.3.2	NO(A)EL	900 ppm equivalent to 69.0 mg/kg/day in males and 78.3 mg/kg/day in females	
5.3.3	Other		
5.3.4	Reliability	1	**X**
5.3.5	Deficiencies	No	

Evaluation by Competent Authorities

EVALUATION BY RAPPORTEUR MEMBER STATE

Date	*July 2021*
Materials and Methods	*Applicant version is accepted.*
Results and discussion	*Applicant version is accepted*
Conclusion	*LO(A)EL males: 322 mg BIT/kg bw/day; LO(A)EL females: 356 mg BIT/kg bw/day* *NO(A)EL males: 69 mg BIT/kg bw/day; NO(A)EL females: 78 mg BIT/kg bw/day* *Other conclusions: Other applicant's conclusions are adopted.*

Section A6 Toxicological and Metabolic Studies

Subsection A6.4.1/1 Repeated dose toxicity

Annex Point IIA VI.6.4.1.a/01 **SUBCHRONIC ORAL TOXICITY TEST IN RATS (90 DAYS)**

Reliability	*2 (some parameters of the most recent versions of the guide are missing)*
Acceptability	*Acceptable*
Remarks	*There are some minor mistakes in the following tables. These mistakes are:* *a) Table A6_4_1(1)-7 and Table A6_4_1(1)-8 express that all dietary concentrations of [redacted] were 0 ppm when they obviously were 0, 200, 900 and 4000 ppm;* *b) Table A6_4_1(1)-14 states the dose level of [redacted] in mg/kg/day when they are really expressed in ppm;* *c) The increase in plasma alkaline phosphatase was detected in males dosed with 4000 ppm instead in females, as is stated in Table A6_4_1(1)-14.*

Table A6_4_1(1)-1: Treatment Groups, Average Body Weights and Animal Identification Numbers

Group	Treatment (mg [redacted] kg/day)	Average Body Weight (g), SD*		Experimental Numbers	
		Males	Female	Males	Female
1	0	192.2, 19.1	146.9, 9.0	1-12	13-24
2	200	184.3, 17.8	150.3, 8.7	25-36	37-48
3	900	185.5, 15.3	148.9, 7.3	49-60	61-72
4	4000	187.9, 18.8	145.0, 7.1	73-84	85-96

* Recorded during week 1.

Table A6_4_1(1)-2: Mean dose of [redacted] Received (mg/kg/day) over the 90 day Dosing Period

Week of Treatment	Dietary Concentration of [redacted]					
	200 ppm		900 ppm		4000 ppm	
	Male	Female	Male	Female	Male	Female
1	24.44	24.25	110.00	110.34	465.86	493.93
2	21.14	22.20	95.12	97.20	461.97	451.87
3	18.89	20.10	84.51	88.85	406.29	408.33
4	17.01	18.47	75.19	81.24	348.55	376.00
5	15.76	18.15	72.60	81.18	329.32	377.32
6	14.48	16.94	67.67	76.68	305.82	344.56
7	13.81	16.82	62.08	73.02	292.27	318.47
8	13.22	16.91	59.17	70.27	287.63	324.75
9	12.90	15.73	57.26	70.33	278.98	318.42
10	12.32	15.01	55.87	69.37	261.92	299.94
11	12.02	15.35	54.80	67.53	263.44	304.35
12	11.50	15.18	51.63	67.59	240.23	320.39
13	11.25	14.07	50.60	63.95	243.88	293.31
Mean	15.29	17.63	68.96	78.27	322.00	356.28

Table A6_4_1(1)-3: Intergroup Comparison of Body Weights Adjusted for Initial Weight - Male

Week	Dietary Concentration of [redacted] (ppm)			
	0 (Control)	200	900	4000
	Weight (g), SD			
1	192.2	184.3	185.5	187.9
2	239.3	241.2	234.4*	224.8**
3	283.8	284.8	273.3**	268.1**
4	316.1	319.2	307.3	301.1**
5	343.4	349.3	331.6*	321.9**
6	369.1	375.2	356.7	345.8**
7	390.6	396.4	377.9	365.7**
8	400.5	410.7	391.0	380.8*
9	414.0	426.4	403.7	390.4**
10	429.4	443.5	417.2	406.7**
11	442.6	455.3	426.1	418.4**
12	454.5	463.2	436.8	427.9**
13 (Start)***	460.7	468.4	439.9*	431.7**
13 (End)***	463.3	471.5	448.0	433.9**

* Statistically significant difference from the control group mean at the 5% level (Student's t-test, two-sided).
** Statistically significant difference from the control group mean at the 1% level (Student's t-test, two-sided).
*** Body weights were recorded at the beginning and end of week 13.

Table A6_4_1(1)-4: Intergroup Comparison of Body Weights Adjusted for Initial Weight - Female

Week	Dietary Concentration of [redacted] (ppm)			
	0 (Control)	200	900	4000
	Weight (g)			
1	146.9	150.3	148.9	145.0
2	170.5	170.2	173.1	166.9
3	191.0	189.4	191.7	181.6*
4	202.6	200.3	201.8	193.2*
5	213.3	212.0	211.1	204.0**
6	226.6	223.1	220.0*	211.9**
7	232.2	226.3	227.1	220.1**
8	233.8	234.2	230.3	223.6*
9	240.1	243.2	233.8	226.4**
10	248.1	251.3	242.2	235.6**
11	250.4	250.5	244.6	234.6**
12	251.1	251.3	247.3	237.8**
13 (Start)***	254.8	258.9	257.0	245.1*
13 (End)***	259.0	258.4	255.8	244.7**

* Statistically significant difference from the control group mean at the 5% level (Student's t-test, two-sided).
** Statistically significant difference from the control group mean at the 1% level (Student's t-test, two-sided).
*** Body weights were recorded at the beginning and end of week 13.

Table A6_4_1(1)-5: Intergroup Comparison of Food Consumption - Male

Week	Dietary Concentration of [redacted] (ppm)			
	0 (Control)	200	900	4000
	Food Consumption (g/rat/day), SD			
1	26.6, 1.7	25.8, 1.0	25.5, 1.1	24.1*, 2.6
2	28.5, 2.0	27.5, 1.6	26.7, 0.7	28.5, 3.4
3	28.5, 1.7	28.2, 1.6	27.1, 0.9	29.0, 2.2
4	28.7, 1.6	28.1, 1.9	26.5*, 0.4	27.2, 1.2
5	29.6, 1.7	28.2, 1.0	27.5*, 1.1	27.5*, 1.4
6	28.6, 0.9	27.6, 1.3	27.4, 1.0	27.3*, 1.0
7	27.4, 0.7	27.5, 0.7	26.3, 0.3	27.3, 0.8
8	27.3, 0.5	27.4, 0.9	25.9*, 0.6	27.8, 1.1
9	27.6, 0.9	27.8, 1.0	25.9, 0.4	27.8, 1.8
10	27.8, 1.2	27.4, 1.5	26.0*, 0.7	27.1, 0.8
11	27.7, 0.9	27.3, 0.9	26.1, 0.9	27.9, 1.5
12	27.1, 0.9	26.5, 0.6	25.0**, 0.8	27.9, 0.5
13	26.2, 1.0	26.2, 0.6	24.8, 0.7	26.4, 1.3
Total (Weeks 1-13)	2531.9, 88.8	2487.9, 89.2	2385.4**, 56.6	2475.9, 94.2

SD = Standard Deviation
* Statistically significant difference from the control group mean at the 5% level (Student's t-test, two-sided).
** Statistically significant difference from the control group mean at the 1% level (Student's t-test, two-sided).

Table A6_4_1(1)-6: Intergroup Comparison of Food Consumption - Female

Week	Dietary Concentration of [redacted] (ppm)			
	0 (Control)	200	900	4000
	Food Consumption (g/rat/day), SD			
1	19.3, 1.2	19.6, 0.1	19.8, 0.2	19.1, 2.0
2	20.0, 1.5	20.2, 0.3	19.8, 1.2	19.4, 1.0
3	19.7, 1.2	19.9, 0.1	19.5, 0.3	18.8, 1.8
4	19.8, 0.7	19.3, 0.5	18.8, 0.6	18.4*, 0.3
5	20.6, 0.6	20.0, 0.2	19.5*, 0.6	19.3*, 0.4
6	19.6, 1.2	19.3, 0.7	19.2, 1.4	18.3., 0.4
7	18.9, 0.7	19.6, 1.2	18.7, 0.8	17.4, 1.0
8	19.6, 0.5	20.4, 0.8	18.2, 0.9	18.0*, 0.9
9	19.3, 0.6	19.6, 0.4	18.7, 0.4	18.2*, 0.2
10	18.6, 0.5	19.0, 0.6	18.9, 0.6	17.4*, 0.6
11	18.5, 0.7	19.5*, 0.3	18.6, 0.5	17.7, 0.1
12	19.0, 0.7	19.6, 0.7	19.0, 0.8	19.1, 0.2
13	18.5, 1.3	18.4, 0.9	18.3, 0.1	17.7, 1.1
Total (Weeks 1-13)	1760.4, 73.0	1730.8, 33.1	1728.9, 55.8	1671.7, 35.2

SD = Standard Deviation
* Statistically significant difference from the control group mean at the 5% level (Student's t-test, two-sided).

Table A6_4_1(1)-7: Intergroup Comparison of Food Utilisation - Male

Week	Dietary Concentration of [redacted] (ppm)			
	0 (Control)	0 (Control)	0 (Control)	0 (Control)
	Food Utilisation (g growth/ 100 g food)			
1-4	20.08, 0.75	20.90, 1.28	19.38, 1.60	17.75**, 1.22
5-8	8.97, 1.61	9.92, 0.36	9.61, 0.03	8.91, 0.60
9-13	5.15, 0.37	4.79, 0.52	4.96, 0.27	4.61, 0.39
Overall (1-13)	10.98, 0.52	11.36, 0.65	10.90, 0.52	9.97**, 0.18

** Statistically significant difference from the control group mean at the 1% level (Student's t-test, two-sided).

Table A6_4_1(1)-8: Intergroup Comparison of Food Utilisation - Female

Week	Dietary Concentration of [redacted] (ppm)			
	0 (Control)	0 (Control)	0 (Control)	0 (Control)
	Food Utilisation (g growth/ 100 g food)			
1-4	11.85, 0.73	11.64, 1.45	11.61, 1.08	10.61, 0.49
5-8	4.88, 0.45	5.57, 1.03	4.27, 0.62	4.41, 0.68
9-13	2.83, 0.86	2.36, 0.67	3.41, 0.74	2.79, 1.12
Overall (1-13)	6.30, 0.45	6.24, 0.56	6.27, 0.49	5.76, 0.43

Table A6_4_1(2)-9: Intergroup Comparison of Organ Weights - Adrenals

Adrenals		Dietary Concentration of [redacted]			
		0	200	900	4000
Males					
Terminal Body Weight (g)	Mean	470.0	467.0	445.3	434.6
	SD	27.7	42.0	37.0	32.5
	N	12	12	12	12
Organ Weight (g)	Mean	0.068	0.070	0.066	0.070
	SD	0.010	0.014	0.013	0.009
	N	12	12	12	12
Organ to Body Weight Ratio (%)	Mean	0.015	0.015	0.014	0.016
	SD	0.002	0.003	0.003	0.003
	N	12	12	12	12
Organ Weight Adjusted for Bodyweight		0.068	0.069	0.064	0.071
Females					
Terminal Body Weight (g)	Mean	258.0	261.4	257.2	241.3
	SD	19.7	20.3	15.9	14.5
	N	12	12	12	12
Organ Weight (g)	Mean	0.078	0.090*	0.084	0.070
	SD	0.012	0.014	0.012	0.014
	N	12	12	12	12
Organ to Body Weight Ratio (%)	Mean	0.031	0.035	0.033	0.029
	SD	0.006	0.006	0.005	0.006
	N	12	12	12	12
Organ Weight Adjusted for Bodyweight		0.078	0.091*	0.085	0.069

* Statistically significant difference from the control group mean at the 5% level (Student's t-test, two-sided).

Table A6_4_1(2)-10: Intergroup Comparison of Organ Weights - Brain

Brain		Dietary Concentration of [redacted]			
		0	200	900	4000
Males					
Terminal Body Weight (g)	Mean	470.0	467.0	445.3	434.6
	SD	27.7	42.0	37.0	32.5
	N	12	12	12	12
Organ Weight (g)	Mean	2.07	2.07	2.04	2.00**
	SD	0.06	0.08	0.09	0.05
	N	12	12	12	12
Organ to Body Weight Ratio (%)	Mean	0.44	0.45	0.46	0.46
	SD	0.02	0.03	0.02	0.03
	N	12	12	12	12
Organ Weight Adjusted for Bodyweight		2.05	2.05	2.05	2.02
Females					
Terminal Body Weight (g)	Mean	258.0	261.4	257.2	241.3
	SD	19.7	20.3	15.9	14.5
	N	12	12	12	12
Organ Weight (g)	Mean	1.89	1.89	1.89	1.86
	SD	0.05	0.05	0.05	0.04
	N	12	12	12	12
Organ to Body Weight Ratio (%)	Mean	0.74	0.73	0.74	0.77
	SD	0.05	0.05	0.04	0.04
	N	12	12	12	12
Organ Weight Adjusted for Bodyweight		1.89	1.88	1.88	1.88

Table A6_4_1(2)-11: Intergroup Comparison of Organ Weights - Kidneys

Kidneys		Dietary Concentration of [redacted]			
		0	200	900	4000
Males					
Terminal Body Weight (g)	**Mean**	470.0	467.0	445.3	434.6
	SD	27.7	42.0	37.0	32.5
	N	12	12	12	12
Organ Weight (g)	**Mean**	3.15	3.21	2.95	3.01
	SD	0.24	0.41	0.34	0.25
	N	12	12	12	12
Organ to Body Weight Ratio (%)	**Mean**	0.67	0.69	0.66	0.69
	SD	0.05	0.08	0.05	0.03
	N	12	12	12	12
Organ Weight Adjusted for Bodyweight		3.04	3.12	3.01	3.15
Females					
Terminal Body Weight (g)	**Mean**	258.0	261.4	257.2	241.3
	SD	19.7	20.3	15.9	14.5
	N	12	12	12	12
Organ Weight (g)	**Mean**	1.88	1.92	1.89	1.75*
	SD	0.13	0.11	0.12	0.11
	N	12	12	12	12
Organ to Body Weight Ratio (%)	**Mean**	0.73	0.74	0.74	0.73
	SD	0.05	0.04	0.05	0.04
	N	12	12	12	12
Organ Weight Adjusted for Bodyweight		1.86	1.88	1.88	1.83

Table A6_4_1(2)-12: Intergroup Comparison of Organ Weights-Liver

Liver		Dietary Concentration of [redacted]			
		0	200	900	4000
Males					
Terminal Body Weight (g)	Mean	470.0	467.0	445.3	434.6
	SD	27.7	42.0	37.0	32.5
	N	12	12	12	12
Organ Weight (g)	Mean	19.2	18.5	18.6	17.9
	SD	2.4	1.1	2.0	1.8
	N	12	12	12	12
Organ to Body Weight Ratio (%)	Mean	4.1	4.0	4.2	4.1
	SD	0.5	0.3	0.4	0.3
	N	12	12	12	12
Organ Weight Adjusted for Bodyweight		18.4	17.9	19.1	18.9
Females					
Terminal Body Weight (g)	Mean	258.0	261.4	257.2	241.3
	SD	19.7	20.3	15.9	14.5
	N	12	12	12	12
Organ Weight (g)	Mean	10.0	10.1	9.9	9.4
	SD	0.8	1.3	0.6	0.7
	N	12	12	12	12
Organ to Body Weight Ratio (%)	Mean	3.9	3.8	3.9	3.9
	SD	0.1	0.3	0.3	0.3
	N	12	12	12	12
Organ Weight Adjusted for Bodyweight		9.8	9.8	9.8	9.9

Table A6_4_1(2)-13: Intergroup Comparison of Organ Weights - Testes

Testes		Dietary Concentration of [redacted]			
		0	200	900	4000
Terminal Body Weight (g)	Mean	470.0	467.0	445.3	434.6
	SD	27.7	42.0	37.0	32.5
	N	12	12	12	12
Organ Weight (g)	Mean	3.45	3.41	3.28*	343
	SD	0.24	0.34	0.30	0.22
	N	12	12	12	12
Organ to Body Weight Ratio (%)	Mean	0.74	0.73	0.74	0.79
	SD	0.04	0.07	0.06	0.07
	N	12	12	12	12
Organ Weight Adjusted for Bodyweight		3.44	3.40	3.29*	3.44

Table A6_4_1(1)-14: Summary of Results - 90 Day Oral Toxicity of [redacted] to Rats

Parameter		Dose Level of [redacted] (mg/kg/day)							
		Control		200		900		4000	
		Male	Female	Male	Female	Male	Female	Male	Female
number of animals examined		12	12	12	12	12	12	12	12
number of mortalities		0	0	0	0	0	0	0	0
clinical signs – clinical observations and veterinary observations		-	-	-	-	-	-	-	-
body weight		-	-	-	-	↓[2]	-	↓[1]	↓[1]
food consumption		-	-	-	-	-	-	-	↓[3]
Food utilisation								↓[4]	↓[4]
Ophtalmoscopic examination		-	-	-	-	-	-	-	-
clinical chemistry	plasma alkaline phosphatase	-	-	-	-	-	-	-	↑[5]
	Plasma cholestrol	-	-	-	-	-	↑[6]	-	↑[6]
Haematology		-	-	-	-	-	-	-	-
organ weight		-	-	-	-	-	-	-	-
gross pathology (thickened limiting ridge in the stomach)[7]		-	-	-	-	1[8]	1[8]	10	10
microscopic pathology	Hyperplasia of forestomach adjacent to limiting ridge[9]	-	-	-	-	-	-	11	11
	Mixed inflammatory cell submucosal infiltrate[9]	-	-	-	-	-	-	2	6
	Ulceration with submucosal haemorrhage in forestomach[9]	-	-	-	-	-	-	-	1

	Focal hyperplasia in the oeosophagus[9]	-	-	-	-	-	-	1	-

1: Final body weights of males and females given 4000 ppm were statistically significantly reduced (reduction of 6%) when compared to their respective control values after adjustment for initial body weight.
2: Final body weights of males given 900 ppm were 3% lower than their respective control values after adjustment for initial body weight (this difference was not statistically significant).
3: Evidence of reduction in food consumption in the 4000 ppm female dose group during weeks 4, 5, 8, 9 and 10, this difference was statistically significant at the 1% level when compared to the control group mean.
4: Overall reduction in food utilisation observed in males and females (not statistically significant in females) given 4000ppm. This was considered to be a result of reduced food utilisation which was apparent during the first 4 weeks of the study.
5: Statistically significant increase in plasma alkaline phosphatase when compared to the control group.
6: Statistically significant increase in plasma cholesterol when compared to the control group.
7: A full macroscopic examination was performed on all animals and there were no abnormalities observed except for a thickened limiting ridge in the stomach. The number of animals with this condition is presented.
8: Extra sections of stomach from the block and wet tissue were examined from the male and female given 900 ppm reported as having thickened limiting ridges in the stomach at macroscopic examination. These were normal on microscopic examination.
9: Histopathological examination of all tissues (apart from the stomach, lung, kidney and liver which were examined from all animals) was confined to the control and the 4000 ppm groups. The number of animals with abnormalities is presented

Figure A6_4_1(1)-1: Group Mean Body Weight Recorded Throughout Dosing (Male)

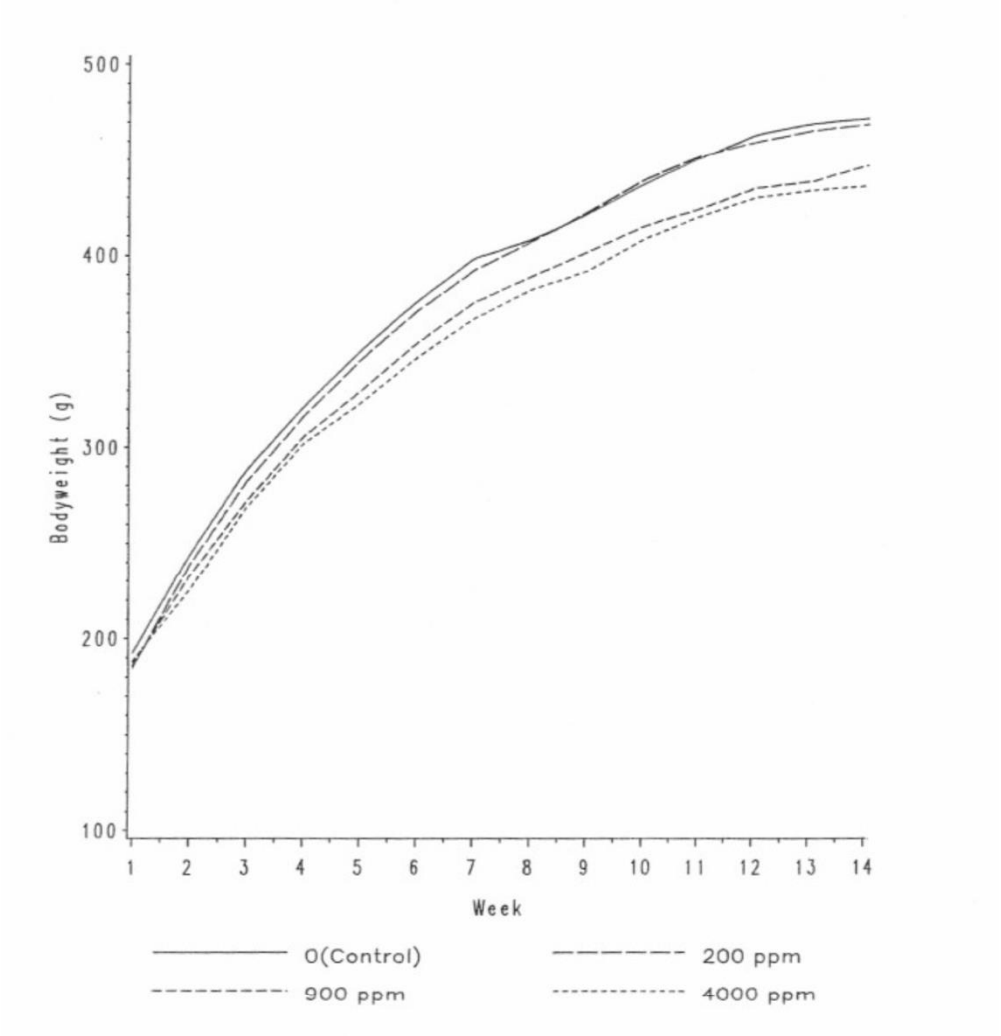


Figure A6_4_1(1)-2: Group Mean Body Weight Recorded Throughout Dosing (Female)

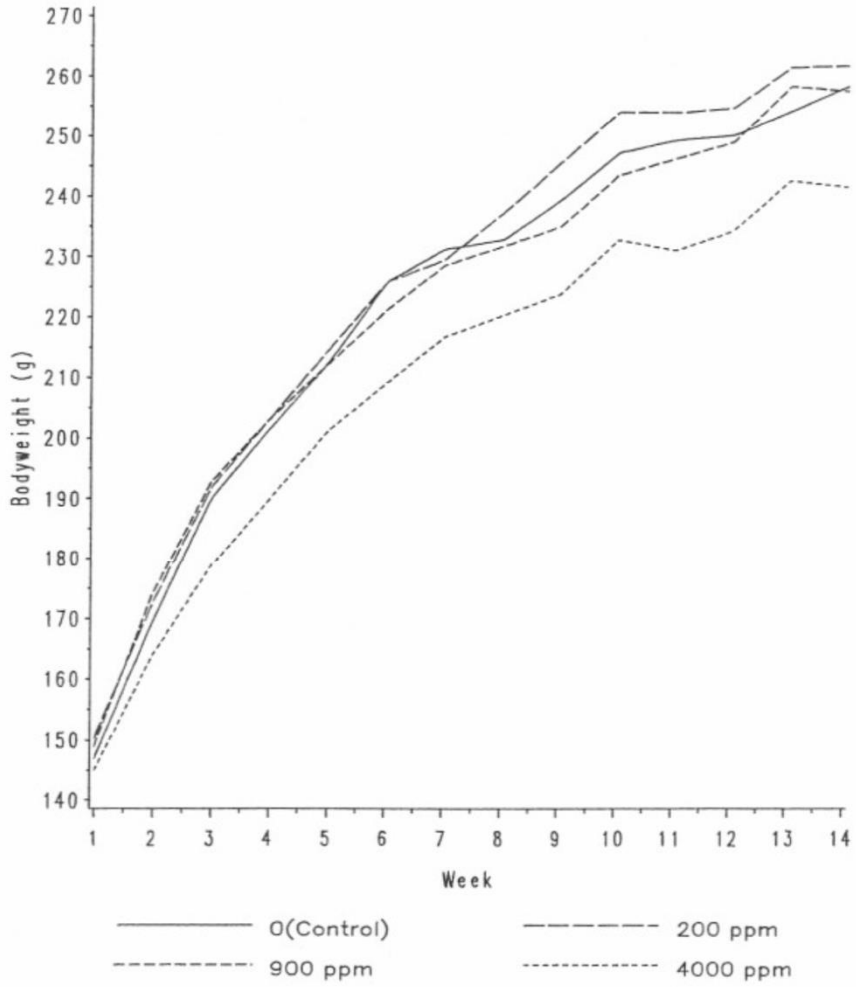
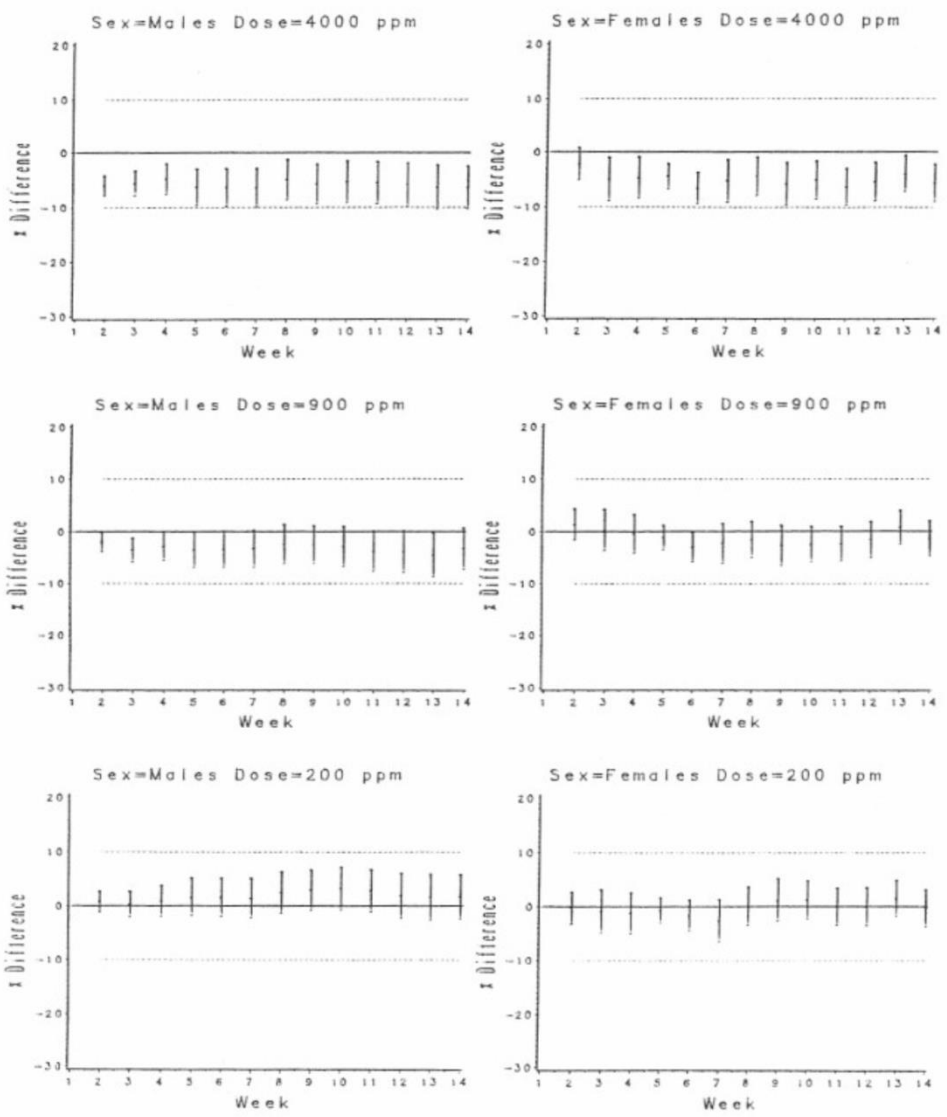


Figure A6_4_1(1)-3: Statistical Analysis of Bodyweights Adjusted for Initial Weight (%Difference from Control Group)



Graphical representation of differences from control based on the analysis of bodyweight adjusted for initial weight and food consumption. Each bar represents the mean percentage difference between control and treated group least square means; the top and bottom of each bar represents the upper and lower 95% confidence limits. If the bar does not cross the zero difference line at a particular week, there is a statistically significant difference between the treated group and the control at that week.

Final body weights of males and females given 4000 ppm were statistically significantly reduced (reduction of 6%) when compared to their respective control values after adjustment for initial body weight. Final body weights of males given 900 ppm were 3% lower than their respective control values after adjustment for initial body weight (not statistically significant).

Section A6 Toxicological and Metabolic Studies

Subsection A6.4.1/2 Repeated dose toxicity

Annex Point IIA VI.6.4.1.b/01 **SUBCHRONIC ORAL TOXICITY TEST IN DOGS (90 DAYS)**

			Official use only
		1 REFERENCE	
1.1	**Reference**	[redacted] 1991; [redacted] 90 Day Oral Toxicity Study in Dogs. [redacted] Report No. [redacted] P/3399	
1.2	**Data protection**	Yes	
1.2.4	Data owner	Arch Chemicals Inc	
1.2.5	Company with letter of access	Clariant Production UK Ltd and Thor GmbH	
1.2.6	Criteria for data protection	Data on existing substance for first entry into Annex I.	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	The study is considered to be compatible with the procedures specified in EC B.27.	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	[redacted]	
3.1.2	Specification	Deviating from specification given in section 2 as follows: The Test Substance employed was pre-dried technical grade active substance.	
3.1.2.2	Purity	94.6% initially, 93.3% post-study	
3.1.2.3	Stability	The study was performed between January 1991 and April 1991. The test material was received with a certificate of analysis (issued 28 September 1990) which stated a purity of 94.6%. The percentage purity was confirmed as 94.6% by analysis performed on 08 October 1990 by the testing laboratory. The batch of [redacted] used in the study was recertified with a purity of 93.3% in May 1991.	

Section A6 Toxicological and Metabolic Studies

Subsection A6.4.1/2 Repeated dose toxicity

Annex Point IIA VI.6.4.1.b/01 **SUBCHRONIC ORAL TOXICITY TEST IN DOGS (90 DAYS)**

3.2	**Test Animals**	
3.2.1	Species	Dog
3.2.2	Strain	Alderley Park Beagle
3.2.3	Source	[redacted] At the Breeding Unit the dogs were vaccinated against canine viral hepatitis, distemper, leptospirosis and canine parvovirus. At the Breeding Unit and during the acclimatisation period at the testing laboratory, all dogs received regular treatment for possible nematode and ear mite infestation.
3.2.4	Sex	Male and female
3.2.5	Age/weight at study initiation	23-25 weeks old. The dogs were 19-20 weeks on arrival and were acclimatised to the [redacted] doghouse for 4 to 5 weeks before dosing commenced. The average body weights recorded in Week 1 are presented in Table A6_4_1(2)-1.
3.2.6	Number of animals per group	3 dose groups, 8 animals per group, 4 male and 4 female. The animals were uniquely identified and randomly assigned to the control and treatment groups. The randomisation procedure resulted in even distribution of dogs to groups according to litter and body weight. Details of the treatment groups and animal identification numbers are presented in Table A6_4_1(2)-1.
3.2.7	Control animals	1 vehicle control group, 4 male and 4 female.
3.3	**Administration/ Exposure**	Oral
3.3.1	Duration of treatment	90 days
3.3.2	Frequency of exposure	Daily
3.3.3	Post-exposure period	There was no post exposure period.
3.3.4	**Oral**	

Section A6 Toxicological and Metabolic Studies

Subsection A6.4.1/2 Repeated dose toxicity

Annex Point IIA VI.6.4.1.b/01 **SUBCHRONIC ORAL TOXICITY TEST IN DOGS (90 DAYS)**

3.3.4.1	Type	Gelatin capsules The capsules had a 9 mL capacity.
3.3.4.2	Concentration	5, 20 and 50 mg/kg bw/day These dose levels were selected on the basis of the results from a six-week oral dose range finding study performed at the test laboratory.
3.3.4.3	Controls	Corn oil
3.3.4.4	Concentration in vehicle	Based on the most recent body weight and adjusted for purity (94.6%).
3.3.4.5	Total volume applied	3 mL corn oil per capsule
3.3.4.6	Controls	Vehicle controls dosed with gelatin capsules containing 3 mL corn oil.
3.4	**Examinations**	
3.4.1	Observations	
3.1.2.3	Clinical signs	The dogs were observed at least once daily (at dosing) and usually on two other occasions each day, for gross clinical and behavioural abnormalities. Daily records of faecal consistency were made. All dogs were also given a full clinical examination by a veterinarian in Week -1 and prior to termination. The examination included cardiac and pulmonary auscultation and indirect ophthalmoscopy.
3.1.2.4	Mortality	Observations were made at least once daily (at dosing) and usually on two other occasions each day.
3.4.2	Body weight	All dogs were weighed weekly, before feeding, throughout the pre-experimental period, on Day 1 and thereafter at weekly intervals during the treatment period.
3.4.3	Food consumption	Food residues were recorded daily prior to giving the next meal and any residual food discarded. These measurements were made for at least 2 weeks pre-experimentally and throughout the treatment period.
3.4.4	Water consumption	Potable water was supplied *ad libitum.*
3.4.5	Ophthalmoscopic examination	Indirect opthalmoscopy was performed by a veterinarian in Week -1 and prior to termination.
3.4.6	Haematology	Jugular vein blood samples were taken before feeding from all dogs in Weeks -1, 4, 8 and 13 and collected into two tubes, one containing

		EDTA and the other 0.11 M trisodium citrate as anticoagulants. The blood was examined for changes in cytological and haemostatic profile by determination of haemoglobin, haematocrit, red cell count, mean cell volume, mean cell haemoglobin, mean cell haemoglobin concentration, total white cell count and platelet count (using an ELT-800, *Ortho* Diagnostics PLC). A differential white cell count was performed based on the classification of 100 white cells in a Romanowsky-stained blood film and the morphological appearance of red cells examined. Kaolin-cephalin and prothrombin times were measured on a Coag-a-Mate (Organon Teknika).
3.4.7	Clinical chemistry	Jugular vein blood samples were taken before feeding from all dogs in Weeks -1, 4, 8 and 13 and collected into lithium heparin tubes. The following parameters were determined in plasma: alanine transaminase, aspartate transaminase, creatine kinase, alkaline phosphatase and gamma-glutamyl transferase activities; urea, creatinine, glucose, albumin, total protein, cholesterol, triglycerides, total bilirubin, calcium and phosphorus (as phosphate); sodium, potassium and chloride ions (all measured using a KONE specific analyser).
3.4.8	Urinalysis	Not performed
3.5	**Sacrifice and pathology**	
3.5.1	Organ weights	The following organ weights were recorded (the left and right components of paired organs were weighed separately): Adrenal glands, brain, epididymides, kidneys, liver, testes and thyroid glands.

Section A6 Toxicological and Metabolic Studies

Subsection A6.4.1/2 Repeated dose toxicity

Annex Point IIA VI.6.4.1.b/01 **SUBCHRONIC ORAL TOXICITY TEST IN DOGS (90 DAYS)**

3.5.2	Gross and histopathology	The following tissues were removed and examined: Adrenal gland, aorta, bone and marrow (sternum), brain, caecum, cervix, colon, duodenum, epididymis, eye, femur (stored), gall bladder, heart, ileum, jejunum, kidney, liver, lung, lymph node (mesenteric and prescapular), mammary gland, oesophagus, ovary, pancreas, pituitary gland, prostate gland, rectum, salivary gland (submandibular), sciatic nerve, skin, spinal cord, spleen, stomach, testis, thymus, thyroid/parathyroid gland, trachea, bladder, uterus, voluntary muscle (semimembranosus) and any tissue exhibiting abnormalities. All tissues were fixed in neutral buffered formol saline, with the exception of eyes which were fixed in Davidson's fluid and testes, skin and mammary gland which were fixed in Bouin's fluid. All tissues were processed, embedded in paraffin wax, sectioned at 5µm and stained with haematoxylin and eosin (H&E).	
3.5.3	Other examinations	Not applicable	
3.5.4	Statistics	Bodyweights were considered by analysis of covariance on initial bodyweight, separately for males and females. Haematological and blood clinical chemistry data were considered at each time of sampling by analysis of covariance on pre-experimental values. Male and female data were analysed and the results were examined to determine whether any differences between control and treated groups were consistent between sexes. The covariate adjustment was based on the separate sex pre-experimental group means. Values which were considered to be anomalous were excluded from the data. These data included creatine kinase from male 519 (Week 4), female 521 (Week 4) and male 527 (Week 8); gamma glutamyl-transferese from female 502 (Week 4) and platelet count from female 505 (Week 8). Organ weights were considered by analysis of variance and analysis of covariance on final bodyweight, separately for males and females. The data from paired organs were examined for differential effects on left and right components. Analyses of variance and covariance allowed for the replicate structure of the study design. All analyses were carried out using the GLM procedure in SAS (1985). Least square means for each group were calculated using the LSMEAN option in SAS PROC GLM. Unbiased estimates of differences from control were provided by the difference between each treatment group least square mean and the control group least square mean. Differences from control were tested statistically by comparing each treatment group least square mean with the control group least square mean using a two-sided Student's *t*-test, based on the error mean square in the analysis.	

Section A6 Toxicological and Metabolic Studies

Subsection A6.4.1/2 Repeated dose toxicity

Annex Point IIA VI.6.4.1.b/01 **SUBCHRONIC ORAL TOXICITY TEST IN DOGS (90 DAYS)**

3.6	**Further remarks**	None
		4 RESULTS AND DISCUSSION
4.1	**Observations**	
4.1.1	Clinical signs	Treatment with 100 mg [redacted] kg/day induced emesis in all dogs shortly after dosing. As a consequence the dose level was reduced to 50 mg/kg/day for three males and three females after 2-4 days dosing with 100 mg/kg/day. The remaining male and female were given 50 mg/kg/day from Day 1. After the reduction in dose level the incidence of emesis was immediately much reduced. Emesis in males was evenly spread throughout the group, being recorded between 10 to 22 days in individual animals. The incidence in females was similar, emesis being recorded on 6 to 10 days for three animals and 28 days for a fourth animal. Emesis post-dosing was also noted but to a lesser extent in the dogs given 20 mg/kg/day. There was an increased incidence in fluid faeces observed in males in the 50 mg/kg/day dose group when compared to the control group (4, 2, 5, and 16 incidences recorded for the control, 5, 20 and 50 mg/kg/day dose groups, respectively). In females an increase was observed in all dose groups when compared to the control group (1, 7, 11, and 12 incidences of fluid faeces recorded for the control, 5, 20 and 50 mg/kg/day dose groups, respectively). There were no other clinical observations considered to be treatment related.
4.1.2	Mortality	No mortalities were observed at any dose level.
4.2	**Body weight gain**	There were no differences in bodyweight between control and treatment groups. Refer to Figures A6_4_1(2)-1 and A6_4_1(2)-2 for a graphical representation of the body weights recorded throughout the study for males and females, respectively.
4.3	**Food consumption and compound intake**	There was no effect on food consumption. All dogs consumed all the diet offered.
4.4	**Ophtalmoscopic examination**	There were no abnormal opthalmological findings.
4.5	**Blood analysis**	
4.5.1	Haematology	The white blood cell count in females given 20 or 50 mg [redacted] kg/day was elevated in Week 8. In the females given 50

		mg/kg/day this was accompanied by neutrophilia. There were no other effects on the haematology profiles attributable to treatment with [redacted] There were other statistically significant differences from control values but these were sporadic, not seen consistently and considered to be not dose related.
4.5.2	Clinical chemistry	Plasma albumin and total protein levels were reduced throughout the study in males given 20 or 50 mg [redacted] /kg/day. Females given 50 mg/kg/day showed a similar effect, with an isolated reduction in plasma total protein level in Week 13 in females at 20 mg/kg/day. Plasma triglyceride levels were increased throughout the study in both sexes given 50 mg/kg/day and marginally in females given 20 mg/kg/day. There were minor reductions in plasma alanine transaminase activities in both sexes given 20 or 50 mg/kg/day, partly reflecting high values in some control animals and low pre-experimental values for animals in treatment groups. Plasma calcium levels were reduced slightly in two females given 50 mg/kg/day.
4.5.3	Urinalysis	Not performed
4.6	**Sacrifice and pathology**	
4.6.1	Organ weights	There was no evidence of any differential effects on left and right components of paired organs. There was no consistent evidence for a relationship between organ weight and final body weight. Consequently, the analyses were performed with consideration of organ weights only. This was not considered to affect the interpretation of the data. The liver weights of males and females given 50 mg [redacted] kg/day were increased by 17% compared to controls. The liver weights of females given 20 mg/kg/day were increased by 12% compared to controls. There was no evidence of any other treatment-related effects on organ weights. Refer to Tables A6_4_1(2)-2 and A6_4_1(2)-3 for an intergroup comparison of organ weights in males and females, respectively.

4.6.2	Gross and histopathology	Macroscopic Findings A small number of macroscopic findings were recorded. No treatment-related findings were detected. Microscopic Findings A small number of pathological lesions were recorded; these were considered not to be treatment-related.
4.7	**Other**	None

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1	**Materials and methods**	Groups of four male and four female beagle dogs were orally dosed with 0, 5, 20 or 50 mg [redacted] kg/day for at least 90 days. The dose was administered in gelatin capsules using corn oil as a vehicle. A vehicle control group, also consisting of four males and four females was included in the study; these animals were dosed with gelatin capsules containing corn oil only. Clinical and behavioural observations were performed at dosing and usually on two other occasions per day. A full clinical examination by a veterinarian was also performed for all animals in Week -1 and prior to termination. Body weights, food consumption and faecal consistency were monitored during the pre-experimental period and throughout the dosing period. Jugular vein blood samples were collected at Week -1, 4, 8 and 13 and examined for changes in haematological and clinical chemistry profiles. At termination, a post-mortem examination was carried out on all dogs, selected organs were weighed and tissues were examined histopathologically.
5.2	**Results and discussion**	A dose of 100 mg [redacted] kg/day induced emesis in all dogs which received this dose, as a result of which the dose level was reduced to 50 mg/kg/day which is considered to be the maximum achievable dose in the dog by this route. Dogs given 50 mg/kg/day showed a much lower incidence of emesis, although it did continue for the rest of the study. Emesis was observed in 3 males and all females in the 20 mg/kg/day dose group, with a total of 34 and 10 incidences, respectively. There was a greater incidence of emesis observed in the 50 mg/kg/day dose group. All animals in this group exhibited emesis with a total of 70 and 53 incidences in males and females, respectively. There was an increased incidence in fluid faeces observed in males in the 50 mg/kg/day dose group when compared to the control group (4, 2, 5, and 16 incidences recorded for the control, 5, 20 and 50 mg/kg/day dose groups, respectively). In females an increase was observed in all dose groups when compared to the control group (1, 7,

11, and 12 incidences of fluid faeces recorded for the control, 5, 20 and 50 mg/kg/day dose groups, respectively).

There were no differences observed in food consumption or bodyweight between the control and treatment groups.

The white blood cell counts in females given 20 and 50 mg [redacted] kg/day were elevated in Week 8. In the females given 50 mg/kg/day this was accompanied by neutrophilia. There were no other observations in the haematology determinations considered to be treatment related. There were other statistically significant differences from control values but these were sporadic, not seen consistently and considered to be not dose related.

In the clinical chemistry determinations, plasma albumin and total protein levels were reduced throughout the study in males given 20 and 50 mg [redacted] /kg/day. Females given 50 mg/kg/day showed a similar effect, with an isolated reduction in plasma total protein level in Week 13 in females at 20 mg/kg/day. Plasma triglyceride levels were increased throughout the study in both sexes given 50 mg/kg/day and marginally in females given 20 mg/kg/day. There were reductions in plasma alanine transaminase activities in both sexes given 20 and 50 mg/kg/day, partly reflecting high the values in some control animals and low pre-experimental values for animals in treatment groups. Plasma calcium levels were also reduced in females given 50 mg/kg/day, a result of two animals having low values, rather than a group effect.

Changes in plasma albumin, total protein and triglyceride levels are indicative of hepatic involvement. This was reflected in the slightly enlarged livers of dogs given 20 or 50 mg [redacted] /kg/day. However there were no pathological findings in the liver considered by the authors to be of toxicological significance. There was no evidence of any other treatment-related effects on organ weights.

There were a small number of macroscopic findings were recorded however no treatment related findings were detected. During histopathological examinations a small number of pathological lesions were recorded however these were also considered to be unrelated to treatment.

5.3 Conclusion

[redacted] when given to dogs at 20 or 50 mg/kg/day induced emesis and clinical chemistry and liver weight changes without any associated histopathological changes.

The no-effect level in this study was 5 mg/kg/day.

5.3.1 LO(A)EL 20 mg/kg/day

5.3.2 NO(A)EL 5 mg/kg/day

Section A6 Toxicological and Metabolic Studies

Subsection A6.4.1/2 Repeated dose toxicity

Annex Point IIA VI.6.4.1.b/01 **SUBCHRONIC ORAL TOXICITY TEST IN DOGS (90 DAYS)**

5.3.3	Other	Not applicable	
5.3.4	Reliability	1	X
5.3.5	Deficiencies	No	

Evaluation by Competent Authorities

EVALUATION BY RAPPORTEUR MEMBER STATE

Date	*July 2021*
Materials and Methods	*Applicant version is accepted.*
Results and discussion	*Applicant version is accepted.*
Conclusion	*LO(A)EL: 20 mg BIT/kg bw/day (males and females)* *NO(A)EL: 5 mg BIT/kg bw/day(males and females)* *Other conclusions: Other applicant's conclusions are adopted.*
Reliability	*2 (some parameters of the most recent versions of the guide are missing)*
Acceptability	*Acceptable*
Remarks	*There are two minor mistakes in Table A6_4_1(2)-4. The decrease in plasma albumin concentration and in plasma total protein levels in animals dosed with 20 mg BIT/kg bw/day was recorded in males, instead of in female as is indicated.*

Table A6_4_1(2)-1: Treatment Groups, Animal Identification Numbers and Mean Body Weights

Group	Treatment (mg [redacted] kg/day)	Average Body Weight[1] (Kg), SD[2]		Experimental Numbers	
		Males	Female	Males	Female
1	0	10.97, 1.16	10.05, 2.05	501-504	502-508
2	5	11.00, 0.84	9.88, 1.23	509-512	513-516
3	20	10.92, 1.04	9.80, 0.93	517-520	521-524
4	50[3]	11.13, 1.91	9.97, 1.52	525-528	529-532

[1] The average body weights presented are from Week 1
[2] SD = Standard Deviation
[3] Dose level of 100mg [redacted] kg/day was given for up to four days and was reduced to 50 mg/kg/day as emesis was observed in all dogs shortly after dosing.

Table A6_4_1(2)-2: Intergroup Comparison of Organ Weights - Males

Organ	Mean Weight (g), SD and Number of Animals	Dose level of [redacted] (mg/kg/day)			
		0	5	20	50
Adrenals	Mean weight (g)	1.38	1.24	1.34	1.23
	SD	0.19	0.23	0.37	0.16
	N	4	4	4	4
Brain	Mean weight (g)	79.1	77.6	81.7	79.8
	SD	2.9	7.6	3.1	2.6
	N	4	4	4	4
Epdidymides	Mean weight (g)	3.73	3.58	3.75	3.84
	SD	0.54	0.49	1.20	0.60
	N	4	4	4	4
Kidneys	Mean weight (g)	59.5	59.5	59.8	59.7
	SD	4.9	11.0	6.5	4.6
	N	4	4	4	4
Liver	Mean weight (g)	473	477	494	554**
	SD	43	27	10	41
	N	4	4	4	4
Testes	Mean weight (g)	21.5	19.7	20.3	22.9
	SD	3.0	4.2	6.3	3,1
	N	4	4	4	4
Thyroids	Mean weight (g)	0.88	0.83	1.06	1.04
	SD	0.17	0.33	0.14	0.12
	N	4	4	4	4

** Statistically significant difference from the control group mean at the 1% level (Student's t-test, two-sided).

Table A6_4_1(2)-3: Intergroup Comparison of Organ Weights - Females

Organ	Mean Weight (g), SD and Number of Animals	Dose level of [redacted] (mg/kg/day)			
		0	5	20	50
Adrenals	Mean weight (g)	1.27	1.22	1.36	1.42
	SD	0.18	0.23	0.09	0.14
	N	4	4	4	4
Brain	Mean weight (g)	73.6	75.1	74.2	76.2
	SD	3.4	4.4	2.7	2.7
	N	4	4	4	4
Kidneys	Mean weight (g)	49.2	51.5	53.3	52.5
	SD	5.6	3.3	3.9	0.9
	N	4	4	4	4
Liver	Mean weight (g)	379	382	423	445*
	SD	57	17	33	45
	N	4	4	4	4
Thyroids	Mean weight (g)	0.82	0.74	0.23	0.83
	SD	0.08	0.07	0.23	0.07
	N	4	4	4	4

* Statistically significant difference from the control group mean at the 5% level (Student's t-test, two-sided).

Table A6_4_1(2)-4: Summary of Results - 90 Day Oral Toxicity of [redacted] to Dogs

Parameter		Dose Level of [redacted] (mg/kg/day)							
		Control		5		20		50	
		Male	Female	Male	Female	Male	Female	Male	Female
number of animals examined		4	4	4	4	4	4	4	4
number of mortalities		0	0	0	0	0	0	0	0
clinical signs – clinical observations and veterinary observations		-	-	-	-	-	-	-	-
gastrointestinal abnormalities -	emesis	-	-	-	-	↑[1]	↑[1]	↑↑[2]	↑↑[2]
	fluid faeces	-	-	-	↑[3]	-	↑[3]	↑[3]	↑[3]
body weight		-	-	-	-	-	-	-	-
food consumption		-	-	-	-	-	-	-	-
opthalmoscopic examination		-	-	-	-	-	-	-	-
clinical chemistry	plasma albumin	-	-	-	-	-	↓[4]	↓[4]	↓[4]
	total protein levels	-	-	-	-	-	↓[4]	↓[4]	↓[4]
	plasma triglyceride	-	-	-	-		↑[5]	↑[5]	↑[5]
	plasma alanine transaminase activities					↓[6]	↓[6]	↓[6]	↓[6]
	plasma calcium								↓[7]
haematology		-	-	-	-	-	↑[8]	-	↑[8]
organ weight (liver)							↑[9]	↑[9]	↑[9]
gross pathology		-	-	-	-	-	-	-	-
microscopic pathology		-	-	-	-	-	-	-	-

- = No dose related change.

[1] Male: Total of 34 incidences of emesis exhibited in 3 animals. Female: Total of 10 incidences of emesis exhibited in 4 animals.

[2] Male: Total of 70 incidences of emesis exhibited in 4 animals. Female: Total of 53 incidences of emesis exhibited in 4 animals.

[3] Female: Increased incidence in fluid faeces observed in all dose groups when compared to the control group (1, 7, 11, and 12 incidences recorded for the control, 5, 20 and 50 mg/kg/day dose groups, respectively). Male: Increased incidence in fluid faeces observed in the 50 mg/kg/day dose group when compared to the control group (4, 2, 5, and 16 incidences recorded for the control, 5, 20 and 50 mg/kg/day dose groups, respectively)

[4] Plasma albumin and total protein levels were reduced throughout the study in males given 20 and 50 mg [redacted] kg/day. Females given 50 mg/kg/day showed a similar effect, with an isolated reduction in plasma total protein level in Week 13 in females at 20 mg/kg/day.

[5] Plasma triglyceride levels were increased throughout the study in both sexes given 50 mg/kg/day and marginally in females given 20 mg/kg/day.

[6] There were minor reductions in plasma alanine transaminase activities in both sexes given 20 or 50 mg [redacted] kg/day, partly reflecting high control values in some control animals and low pre-experimental values for animals in treatment groups.

[7] Plasma calcium levels were reduced slightly in females given 50 mg [redacted] /kg/day, a result of two animals having low values, rather than a group effect.

[8] The white blood cell counts in females given 20 or 50 mg [redacted] /kg/day were elevated in Week 8. In the females given 50 mg/kg/day this was accompanied by neutrophilia.

[9] The liver weights of males and females given 50 mg [redacted] /kg/day were increased by 17% compared to controls. The liver weights of females given 20 mg/kg/day were increased by 12% compared to controls.

Figure A6_4_1(2)-1: Group Mean Body Recorded Throughout Dosing Weight (Male)

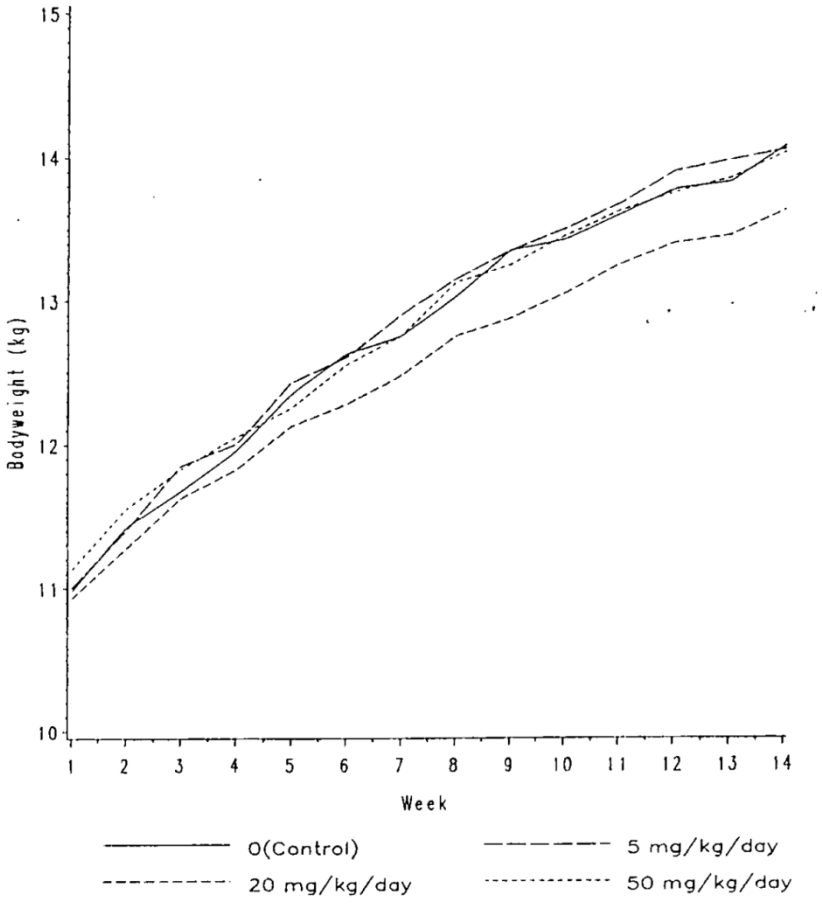
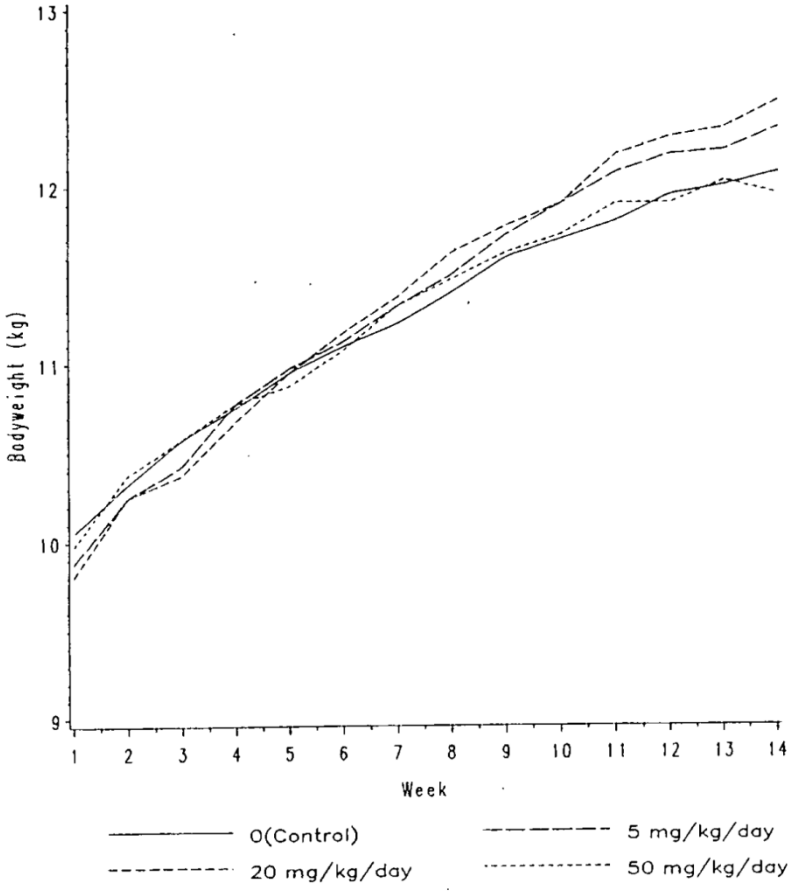


Figure A6_4_1(2)-2: Group Mean Body Weight Recorded Throughout Dosing (Female)



Section A6	**Toxicological and Metabolic Studies**	
Subsection A6.4.2	**Subchronic toxicity test**	
Annex Point IIA VI.6.4.2	**SUBCHRONIC REPEATED DERMAL DOSE TOXICITY**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ]	**Technically not feasible [ ]** **Scientifically unjustified [X]**	
Limited exposure [ ]	**Other justification [ ]**	
Detailed justification:	According to the TNsG on data requirements, A percutaneous study in the rat is preferred, where the potential dermal exposure is significant and route to route extrapolation is not possible. This study is not required on the following basis; • Adequate sub-chronic (oral) toxicity study is available in rodents and dogs (See Section 6.4.1). • Acute dermal toxicity studies showed only minor toxic effects at the highest dose tested (See Section 6.1.2). • It is also possible to calculate the route-to-route exposure from available oral toxicity studies and using dermal penetration studies (Section 6.2) as there are no specific effects observed following dermal exposure in animals. Therefore an accurate and realistic determination of dermal toxicity can be derived from available sub-chronic oral exposure studies and *in vitro* dermal penetration studies.	
Undertaking of intended data submission [ ]		

Evaluation by Competent Authorities

EVALUATION BY RAPPORTEUR MEMBER STATE

Date	*September 2008*
Evaluation of applicant's justification	*Applicant's justification is accepted.*
Conclusion	*Applicant is exempted of the subchronic dermal toxicity test.*
Remarks	

Section A6	**Toxicological and Metabolic Studies**	
Subsection A6.4.3	**Subchronic toxicity test**	
Annex Point IIA VI.6.4.3	**SUBCHRONIC REPEATED INHALATION DOSE TOXICITY**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ]	**Technically not feasible [ ]** **Scientifically unjustified [X]**	
Limited exposure [ ]	**Other justification [ ]**	
Detailed justification:	According to the TNsG on data requirements, a subchronic inhalation toxicity test is required for volatile substances (vapour pressure > 1 x 10^{-2} Pa) or in cases where the potential inhalation exposure is significant. This study is not required on the following basis; • BIT is not a volatile substance. • Based on the intrinsic properties of the test substance (See Justification for non-submission of data for Section 6.1.3), inhalation exposure is not expected, and technically unfeasible to simulate in laboratory tests. • It is also possible to calculate the route-to-route (systemic) exposure from available oral toxicity studies, therefore an accurate and realistic determination of inhalation toxicity can be derived from available sub-chronic oral exposure studies.	
Undertaking of intended data submission [ ]		
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*September 2008*	
Evaluation of applicant's justification	*Applicant's justification is accepted.*	
Conclusion	*Applicant is exempted of the subchronic inhalation toxicity test.*	
Remarks		

Section A6	**Toxicological and Metabolic Studies**	
Subsection A6.5	**Chronic Toxicity**	
Annex Point IIA VI.6.5		
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ]	**Technically not feasible [ ]** **Scientifically unjustified [X]**	
Limited exposure [ ]	**Other justification [ ]**	
Detailed justification:	This justification for non-submission of data is a brief outline of the detailed discussion presented in the following Expert Report; [redacted] Review of toxicological Data on 1,2 Benzisothiazolin 3 one (BIT) and other isothiazolinones; Report: SJB/BIT/150507. The full report is presented in the BIT Dossier IVA6.5. **Subchronic Toxicity of Isothiazolinones** Isothiazolinone derivatives are consistent qualitatively in their toxicological profile. The primary effects from exposure to multiple species are a slight reduction in body weight gain, inflammation at the initial site of contact regardless of the route of exposure, and slight increase in organ weight (liver and kidney). The increase in liver and kidney weight is of questionable toxicological significance since there was no associated histopathological change in these organs. Emesis was observed in the species that has this ability, but it is likely associated with a local irritant effect. **Summary of Genotoxicity of BIT** In the Ames assay, isolated increases in revertants were observed in some Salmonella strains, although the lack of reproducibility and dose response usually seen suggests that this is not a true chemically mediated effect. This is supported by the negative response seen in the L5178Y mammalian cell gene mutation assay. Negative responses were also seen in vitro in a cell transformation assay and a UDS assay. BIT produced a negative response in the in vivo mouse bone marrow micronucleus assay and the rat-liver UDS assay, indicating that the compound is not metabolized to a mutagenic species in the whole animal. BIT did produce chromosomal aberrations in two in vitro cell systems, but the lack of chromosomal aberrations observed in vivo indicates that chromosomal damage would not occur in the whole animal. The conclusion, based on the results from this battery of assays, is that BIT presents no potential to produce genetic damage to mammalian cells in vivo. The lack of potential of BIT to induce genetic damage is similar to other isothiazolinones. These isothiazolinones have been evaluated for genotoxic potential in a number of short term assays including end points of gene mutation, chromosomal damage and DNA repair. **Summary of Metabolism and Disposition of Isothiazolinones** Isothiazolinones, including BIT, are absorbed rapidly from oral	

Section A6	**Toxicological and Metabolic Studies**	
Subsection A6.5	**Chronic Toxicity**	
Annex Point IIA VI.6.5		
	administration and excreted rapidly as well, primarily in the urine. These chemicals are not distributed preferentially to any organ and there is no tendency for bioaccumulation. For those isothiazolinones containing the aromatic ring, the available data indicate that the metabolism follows the path of ring opening with oxidation of the sulphur and methylation of the nitrogen if not already occupied by an alkyl group. For those compounds without the aromatic ring, the isothiazolinone ring undergoes more extensive catabolism. The metabolism of isothiazolinones is rapid and virtually complete with little to no excretion of the parent compound. **Structure-Activity Relationship analysis for BIT** BIT was assessed for carcinogenic potential through structure-activity relationship (SAR) analyses. Based on the results from the 4 models, BIT is predicted to lack the potential to cause cancer. Thus, it has a high probability of not inducing cancer in either rats or mice. The estimate from each model is derived from a structural comparison of BIT to chemicals previously assessed for carcinogenicity. The estimates are robust since none is based on the results of a single model but rather on analyses using Bayes' Theorem to combine the Rat/MIT CMIT and the Mouse/OIT predictions. Moreover, individual predictions are not based on the occurrence of a single descriptor (i.e., fragment) but rather multiple descriptors. And finally, each fragment is derived from several compounds with similar carcinogenic or non-carcinogenic activity. **Summary** The toxicological profile of BIT has been compared to that of other isothiazolinones to demonstrate the similarity in the toxicology for members of this chemical class. Illustration of toxicological similarity between isothiazolinones allows the reasoned judgment that carcinogenicity and chronic toxicity data should not be required for BIT.	
Undertaking of intended data submission [ ]		
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*September 2008*	
Evaluation of applicant's justification	*Applicant's justification is accepted.*	

Section A6	**Toxicological and Metabolic Studies**
Subsection A6.5	**Chronic Toxicity**
Annex Point IIA VI.6.5	
Conclusion	*Applicant is exempted of the chronic toxicity study.*
Remarks	

Section A6	**Toxicological and Metabolic Studies**
Subsection A6.6.1/1	***In vitro* gene mutation study in bacteria**
Annex Point IIA VI.6.6.1	**Bacterial Reverse Mutation Test (S. *typhimurium)***

			Official use only
		1 REFERENCE	
1.1	**Reference**	[redacted] 1989; [redacted] An Evaluation in the Salmonella Mutation Assay. [redacted] Report No. [redacted] P/2369	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Arch Chemicals Inc	
1.2.2	Company with letter of access	Clariant Production UK Ltd and Thor GmbH	
1.2.3	Criteria for data protection	Data on existing substance for first entry in to Annex I.	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	OECD 471 (1983), UKEMS 1983. Considered largely compatible with the current OECD 471.	
2.2	**GLP**	Yes	
2.3	**Deviations**	See 3.2.1, 3.2.4, 5.3.2	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	[redacted]	
3.1.2	Specification	As given in section 2	
3.1.2.1	Description	73.4% w/w	

Section A6 Toxicological and Metabolic Studies

Subsection A6.6.1/1 *In vitro* gene mutation study in bacteria

Annex Point IIA VI.6.6.1 **Bacterial Reverse Mutation Test (S. *typhimurium)***

3.1.2.2	Purity	The test item is stable under the storage conditions used in this study (information supplied by the Sponsor).
3.1.2.3	Stability	Bacterial reverse mutation test
3.2	**Study Type**	*S. typhimurium*: TA98, TA100, TA1535, TA1537, TA1538
3.2.1	Organism/cell type	As given in section 2
3.2.2	Deficiencies / Proficiencies	Histidine amino acid deficient
3.2.3	Metabolic activation system	S9 mix; rat, liver, induced, Aroclor 1254, 500 mg/kg i.p.
3.2.4	Positive control	2-Aminoanthracene (2AA), +S9, TA98, TA100, TA1535, TA1537, TA1538 Daunomycin HCl (DR), -S9, TA98 *N*-Methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG), -S9, TA100 and TA1535 Acridine Mutagen ICR191, -S9, TA1537 4-Nitro-*o*-phenylenediamine (4NPD), -S9, TA1538
3.3	**Application of test substance**	
3.3.1	Concentrations	0.32, 1.6, 8.0, 40, 100, 200 µg/plate +S9 0.064, 0.32, 1.6, 8.0, 40, 80 µg/plate -S9
3.3.2	Way of application	Plate incorporation, dimethylsulphoxide (DMSO) solvent
3.3.3	Pre-incubation time	Not applicable
3.3.4	Other modifications	Not applicable
3.4	**Examinations**	Not applicable
	4	**RESULTS AND DISCUSSION**
4.1	**Genotoxicity**	
4.1.1	without metabolic activation	No.

4.1.2	with metabolic activation	No.
4.2	**Cytotoxicity**	Yes, +S9 @ 200 µg/plate, -S9 @ 80 µg/plate

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and methods

[redacted] was evaluated in the bacterial mutagenicity assay, using five strains of *Salmonella typhimurium* (TA1535, TAl537, TA1538, TA98 and TA100). The study is considered to be compatible with OECD Guideline 471 (study initiated in 1988 and performed according to OECD Guideline 471, 1983).

A preliminary dose range finding test was performed with strain TA100 in the absence and presence of metabolic activation in the range 5000 to 1.6 µg/plate followed by a preliminary test in the range 200 to 10 µg/plate in strain TA100 only. Given the cytotoxicity of [redacted] observed in the absence and presence of metabolic activation (80 µg/plate and 200 µg/plate, respectively) the main study was conducted with all strains at concentrations of 80 to 0.064 µg/plate without metabolic activation and of 200 to 0.32 µg/plate with metabolic activation.

Each plate was prepared by the 'plate incorporation procedure' where the appropriate components were added to a bijou bottle. These components were the appropriate bacterial strain; S-9 mix (for tests with metabolic activation) or buffer (for tests without metabolic activation); appropriate concentration of [redacted] appropriate chemical for positive controls or DMSO for negative controls; and top agar (10 mL histidine/bioton stock solution:100 mL agar (v/v)). The contents of the bijou bottle were poured immediately onto the surface of a prepared Vogel Bonner plate, allowed to gel and incubated inverted at 37 °C for approximately 66 hours in the dark.

After incubation revertant colonies were counted using an automated colony counter (AMS 40-10 Image Analyser). The test data were analysed for validity and for any reproducible dose related increase in revertant colonies. Statistical analysis was performed using a one tailed Student's t-test.

5.2 Results and discussion

[redacted] induced significant cytotoxicity in all strains tested at concentrations of 80 µg/plate in the absence of metabolic activation and 200 µg/plate in the presence of metabolic activation.

In the absence of metabolic activation. [redacted] did not induce any significant, reproducible increases in the observed numbers of revertant colonies in strains TA100, TA1535 or TA1538. There was one result in each test performed with strain TA98 in the absence of metabolic activation which could be considered indicative of a possible effect. These data however are

not considered to indicate a mutagenic response since the results are not statistically significant and confined to only one dose level in each test. In one experiment performed with TA1537 without metabolic activation there were three results which could be considered indicative of a possible effect. These data are also not considered to indicate a mutagenic response since the results are not statistically significant and confined to only one test.

In the presence of metabolic activation. [redacted] did not induce any significant, reproducible increases in the observed numbers of revertant colonies in strains TA100 or TA1535. In both experiments with metabolic activation, statistically significant responses were observed in strain TA98, reaching a maximum response of 1.6 × background in each case. However these effects were not reproducible in two further experiments with TA98 with metabolic activation. A statistically significant response to [redacted] was observed with strains TA1537 and TA1538 with metabolic activation in the second experiment but not in the first. Although the maximum response observed exceeded 2 × background in both strains, no reproducible effects were obtained in one further experiment with TA1537 or in two experiments with TA1538. This lack of reproducibility indicates that the observed effects, in the presence of metabolic activation, in these three strains are not due to compound induced mutations.

The positive control data for each strain tested showed evidence of a mutagenic response which was dose related in the absence and presence of metabolic activation. The chemicals used for positive control samples therefore induced the expected response, indicating that all strains were behaving appropriately for this reverse mutation assay.

It can be concluded that under the conditions of this assay there was a non-mutagenic response to [redacted] when tested to limit doses of 200 µg/plate (with metabolic activation) and 80 µg/plate (without metabolic activation), at which concentrations significant toxicity was observed in each case.

[It should be noted that in a study performed by the same laboratory [redacted] 1988; [redacted] Report No. [redacted]P/2056) using the same strains of *S. typhimurium* and dose ranges of 300 to 4.0 µg/plate, 250 to 0.8 µg/plate (both with metabolic activation) and 200 to 0.8 µg/plate (without metabolic activation), [redacted] induced reproducible, dose-related, significant increases in the observed numbers of revertant colonies in strains TA1538 and TA98 in the presence of metabolic activation. These effects were not observed in strains TA1535, TA1537 or TA100 in the presence of metabolic activation. In the absence of metabolic activation, [redacted] did not induce any significant reproducible increases in the observed numbers of revertant colonies in any of the five tester strains used.

Section A6 Toxicological and Metabolic Studies

Subsection A6.6.1/1 *In vitro* gene mutation study in bacteria

Annex Point IIA VI.6.6.1 Bacterial Reverse Mutation Test (S. *typhimurium)*

In another study ([redacted] 1995; [redacted]., Report No. 94/NLL051/0788) which tested the mutagenic effects of [redacted] BIT at dose ranges of 100 to 1.0 µg/plate (with and without metabolic activation) on strains TA98, TA100, TA1535 and TA1537, there was no evidence of mutagenic activity under the conditions of the test. [redacted] 1979 ([redacted] Report No. [redacted]R/494) tested strains TA1535, TA1537, TA1538, TA100 and TA98 at a dose range of 2500 to 0.32 µg/plate (with and without metabolic activation) and there was no mutagenic effect observed in the presence or absence of metabolic activation.

When the data from [redacted] (1995) and [redacted] (1979) are considered together with the [redacted] (1989) data (presented in this summary) it can be concluded that the presence of 1,2-Benzisothiazolin-3-one is unlikely to evoke mutagenic activity in this type of assay. The positive result observed in Callander (1988) was not observed in three other studies performed under similar conditions.]

5.3 Conclusion

Under the conditions of this assay there was a non-mutagenic response to [redacted] when tested to limit doses of 200 µg/plate (with metabolic activation) and 80 µg/plate (without metabolic activation), at which concentrations significant toxicity was observed in each case.

5.3.1 Reliability

2

5.3.2 Deficiencies

The study can be considered to be essentially compatible with the current OECD Guideline 471 with the following exceptions:

The chemicals used for the positive controls in the absence of metabolic activation were *N*-Methyl-*N*'-nitro-*N*-nitrosoguanidine (TA100 and TA1535), Acridine mutagen (TA1537), 4-Nitro-*o*-phenylenediamine (TA1538) and Daunomycin HCl (TA98). The current guideline recommends Sodium azide (TA100 and TA1535), 9-Aminoacridine (TA1537) and 2-Nitrofluorine (TA98) in the absence of metabolic activation. The use of some currently non-standard positive control chemicals is not considered to affect the reliability of the generated results since the positive control data for each strain tested showed evidence of a mutagenic response which was dose related in the absence and presence of metabolic activation.

The growth phase and cell density of the cultures used was not specifically reported, however guideline compliance (i.e. late exponential or early stationary phase of growth and approximate cell density of 10^9 cells/mL) is claimed.

Five strains of *S. typhimurium* are recommended in the test guideline. Although five strains were tested, only four of the recommended strains (TA98, TA100, TA1535, TA1537) were

tested, along with TA1538 (TA102, capable of detecting certain mutagens which the other strains cannot, was not tested). As testing of [redacted] in the *in vitro* mammalian cell (mouse lymphoma) gene mutation test and in two *in vivo* (micronucleus and UDS) assays has generated negative results, the absence of *in vitro* gene mutation testing in *S. typhimurium* TA102 is not considered critical overall in the context of the genotoxicity database.

Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*September 2008*
Materials and Methods	*Applicant's version is accepted.*
Results and discussion	*Applicant's version is accepted.*
Conclusion	*Applicant's version is adopted.*
Reliability	*2*
Acceptability	*Acceptable*
Remarks	

Table 6_6_1-1: Experimental Design

Plate	Concentration (µg/plate [redacted])	Replicates
[redacted] with S9 (metabolic activation)	200	3 plates per dose
	100	
	80	
	40	
	8.0	
	1.6	
	0.32	
[redacted] without S9 (without metabolic activation)	80	3 plates per dose
	40	
	8.0	
	1.6	
	0.32	
	0.064	

Plate	Plate Description	Replicates
Negative Controls	Solvent: DMSO (100 µL)	5 plates
	Absolute	2 plates
Positive Controls With S9	2-Aminoanthracene: TA98, TA100, TA1535, TA1537, TA1538	3 Doses for each strain, 2 plates at each dose
Positive Controls Without S9	*N*-Methyl-*N*'-nitro-*N*-nitrosoguanidine: TA100 and TA1535	
	Acridine Mutagen: TA1537	
	4-Nitro-*o*-phenylenediamine: TA1538	
	Daunomycin HCl: TA98	

Section A6 Toxicological and Metabolic Studies

Subsection A6.6.2/1 *In vitro* cytogenicity study in mammalian cells

Annex Point IIA VI.6.6.2 ***In Vitro* Mammalian Chromosome Aberration Test (Human Peripheral Lymphocytes).**

			Official use only
		1 REFERENCE	
1.1	**Reference**	[redacted] 1995; [redacted] BIT: An *in vitro* Test for Induction of Chromosome Damage: Cytogenetic Study in Cultured Human Peripheral Lymphocytes. [redacted] Report No. 95/NLL052/0540	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Clariant Production UK Ltd	
1.2.2	Companies with letter of access	Arch Chemicals Inc and Thor GmbH	
1.2.3	Criteria for data protection	Data on existing substance for first entry into Annex I.	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	EC B.10 (1992), OECD 473 (1983). Considered largely compatible with EC B.10 (2000) and OECD 473 (1997).	
2.2	**GLP**	Yes	
2.3	**Deviations**	See 3.2.4 and 5.3.2	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	[redacted]	
3.1.2	Specification	As given in section 2	
3.1.2.1	Purity	1,2-Benzisothiazol-3-(2*H*)-one (BIT) purity of 98.8% (analysis was done on batch sample not test sample)	
3.1.2.2	Stability	Stable under storage conditions (ambient temperature in the dark)	
3.2	**Study Type**	*In vitro* mammalian chromosome aberration test	
3.2.1	Organism/cell type	Cultured human peripheral lymphocytes Human peripheral blood was obtained by venepuncture from a healthy non-smoking male human volunteer not currently taking	

		any medication, and collected in heparinised vessels. The doubling time for lymphocytes from the donor was assessed in June 1994 and found to be 14.3 hours. Small inocula of whole blood (0.5 mL) were added to tubes containing culture medium and phytohaemagglutinin solution to stimulate lymphocytes to divide. The tubes were sealed and incubated at 37°C with occasional shaking to prevent clumping. After approximately 48 hours of incubation, cultures were centrifuged, the supernatant removed and the cell pellet resuspended in culture medium.
3.2.2	Deficiencies / Proficiencies	Not applicable

Section A6 Toxicological and Metabolic Studies

Subsection A6.6.2/1 *In vitro* cytogenicity study in mammalian cells

Annex Point IIA VI.6.6.2 ***In Vitro* Mammalian Chromosome Aberration Test (Human Peripheral Lymphocytes).**

3.2.3 Metabolic activation system

Liver derived metabolic activation system (S-9 mix), prepared as follows:

Component	Volume (mL)
0.1 M KH_2PO_4-Na_2HPO_4 buffer (pH 7.4)	7.4
0.4 M $MgCl_2.6H_2O$/1 .65 M KC1 aqueous solution	0.2
0.1 M NADP, sodium salt, in aqueous solution	0.4
0. 1 M glucose-6-phosphate, sodium salt, in aqueous solution	0.5
Supernatant from liver homogenate (prepared as detailed below)	1.5

Young male CD rats, approximately 200 g bodyweight, were obtained from [redacted] Aroclor 1254 (500 mg/kg bodyweight in corn oil) was administered as a single intraperitoneal injection to induce microsomal enzyme activity.

Five days after treatment, the animals were sacrificed by cervical dislocation, the livers were removed, washed in cold 0.15 M KC1 and then homogenised with one volume of the same medium. Homogenates were centrifuged and supernatants collected and stored frozen until required for preparation of the S-9 mix (supernatant is used within one year of preparation).

3.2.4 Positive control

With metabolic activation: cyclophosphamide

Without metabolic activation: chlorambucil

Solutions of cyclophosphamide (Endoxana, Asta Medica Ltd.) in sterile water (purified by reverse osmosis), and chlorambucil (Sigma Chemicals) in ethanol, were prepared immediately prior to use and served as positive controls. Cyclophosphamide is converted in the presence of an S-9 mix activation system to a highly reactive, clastogenic form. Chlorambucil is a direct-acting clastogenic agent.

3.3 Application of test substance

Section A6 Toxicological and Metabolic Studies

Subsection A6.6.2/1 *In vitro* cytogenicity study in mammalian cells

Annex Point IIA VI.6.6.2 ***In Vitro*** **Mammalian Chromosome Aberration Test (Human Peripheral Lymphocytes).**

3.3.1	Concentrations	Preliminary toxicity test: 8 to 5000 µg/mL [redacted] BIT (with and without metabolic activation, sampling times of 21 and 45 hours) First cytogenic test: 1 to 32 µg/mL [redacted] BIT (with and without metabolic activation, sampling time of 21 hours), 8 to 32 µg/mL [redacted] BIT (with metabolic activation, sampling time of 45 hours), and 4 to 32 µg/mL [redacted] BIT (without metabolic activation, sampling time of 45 hours) Repeat first cytogenic test: 24 to 40 µg/mL [redacted] BIT (with metabolic activation, sampling time of 45 hours) Second cytogenic test: 2 to 16 µg/mL [redacted] BIT (with and without metabolic activation, sampling time of 21 hours) No correction was made for purity of the test item.

Section A6 Toxicological and Metabolic Studies

Subsection A6.6.2/1 *In vitro* cytogenicity study in mammalian cells

Annex Point IIA VI.6.6.2 ***In Vitro*** **Mammalian Chromosome Aberration Test (Human Peripheral Lymphocytes).**

3.3.2	Way of application	Treatment The test material was dissolved in dimethyl sulphoxide (DMSO) and added to the test cultures immediately. Freshly prepared S-9 mix (1.0 mL) was added to the appropriate cultures (see Section 3.2.1) and an aliquot of test solution or solvent (100 µL) or positive control solution (50 µL) was added to the relevant cultures. The final volume in each culture was 10 mL. Duplicate cultures were established for each treatment in the preliminary toxicity and main cytogenetic tests. All cultures were incubated at 37 °C, in a shaking water bath, for three hours. After this initial exposure period, the cultures treated in the absence of S-9 mix were transferred to an incubator (37 °C) for the remainder of the scheduled exposure period. Cultures treated in the presence of S-9 mix were centrifuged and the cells washed twice with Hanks Balanced Salt solution to remove the test material and S-9 mix. The cells were then resuspended in culture medium (9.5 mL), and the cultures incubated at 37 °C, under static conditions, until scheduled harvesting. Culture Harvesting Three hours before harvesting, cell division was arrested by the addition of the spindle poison, colcemid to each culture. The tubes were capped and left to incubate for a further three hours. The cells were then harvested by low speed centrifugation and the pellets of cells thus collected were resuspended in hypotonic potassium chloride solution for ten minutes, centrifuged again and later fixed in freshly prepared methanol:glacial acetic acid fixative (3:1 v/v). Slide Preparation After two further changes of fixative, the tubes were centrifuged, the supernatant removed and the cell pellet resuspended in a few drops of fresh fixative. Single drops of the cell suspension were transferred to clean, moist, grease-free glass slides, and the slides were left to air-dry. Two or four slides (for the preliminary toxicity test or main cytogenetic tests, respectively) were made from each culture, stained for ten minutes in Giemsa stain, washed in buffer and left to air dry. Permanent mounts were made using DPX mountant after clearing in xylene.
3.3.3	Pre-incubation time	Not applicable
3.3.4	Other modifications	Not applicable
3.4	**Examinations**	

Section A6 Toxicological and Metabolic Studies

Subsection A6.6.2/1 *In vitro* cytogenicity study in mammalian cells

Annex Point IIA VI.6.6.2 ***In Vitro* Mammalian Chromosome Aberration Test (Human Peripheral Lymphocytes).**

3.4.1 Number of cells evaluated

Preliminary Test

1000 lymphocytes per culture were examined using a light microscope and the mitotic index was calculated as the percentage of lymphocytes examined that were in mitosis (at metaphase).

First Cytogenic Test

Mitotic Index

To examine the toxicity of the test material to dividing lymphocytes, approximately 1000 cells were scored and the mitotic index calculated. Initially, mitotic indices were scored from all cultures.

Chromosomal Aberrations

One hundred metaphases were scored from each culture.

On the basis of the mitotic indices and reductions in mean mitotic index for each [redacted] BIT concentration relative to the solvent control values, slides from cultures treated at the following concentrations of [redacted] BIT were selected for the scoring of chromosomal aberrations:

- 21 hour sampling time, with and without S-9 mix: 4, 8 and 16 µg/mL

- 45 hour sampling time, with S-9 mix: 24 µg/mL

Second Cytogenic Test

Mitotic Index

On the basis of the results of the first cytogenetic test, the [redacted] BIT concentrations selected for use in the second cytogenetic test were 2, 4, 8 and 16 µg/mL, with and without S-9 mix (21 hour sampling time). To examine the toxicity of the test material to dividing lymphocytes, approximately 1000 cells were scored and the mitotic index calculated. Initially, mitotic indices were scored from all cultures.

Chromosomal Aberrations

On the basis of mitotic indices and reductions in mean mitotic index for each [redacted] BIT concentration relative to the solvent control values, slides from cultures treated at 2, 4 and 8 µg/mL in the absence of S-9 mix and 4, 8 and 16 µg/mL in the presence of S-9 mix were selected for the scoring of chromosomal aberrations.

Procedure for Determination of Mitotic Index and Chromosomal Analysis

At least two slides from each culture were randomly selected for examination. The slides were examined under a low power (× l0 objective) and those areas judged to be of sufficient technical quality to permit scoring were located and examined under high power (× l00, oil immersion objective).

From 100 metaphases, with 46 centromeres, the following characters were recorded:

- chromosome number

- all chromosomes normal or some aberrant

- specific types and numbers of aberrations.

Section A6 Toxicological and Metabolic Studies

Subsection A6.6.2/1 *In vitro* cytogenicity study in mammalian cells

Annex Point IIA VI.6.6.2 ***In Vitro* Mammalian Chromosome Aberration Test (Human Peripheral Lymphocytes).**

4 RESULTS AND DISCUSSION

4.1 Genotoxicity

4.1.1	without metabolic activation	Yes First Cytogenetic Test (1 to 32 µg/mL [redacted] BIT) In the absence of metabolic activation, treatment with [redacted] BIT produced statistically significant increases in the frequency of metaphases with aberrant chromosomes at concentrations of 8 and 16 µg/mL at the first (21 hours) harvest time, compared to solvent control values ($p < 0.001$, both including and excluding gap-type aberrations). The increases at 16 µg/mL were reproduced in both replicate cultures and exceeded the historical solvent control range (without S-9 mix: 0-10% including gaps and 0-5% excluding gaps). Slides from cultures treated in the absence of S-9 mix for 45 hours were not analysed because [redacted] BIT was clastogenic at the 21 hour sampling time. Second Cytogenic Test (2 to 16 µg/mL [redacted] BIT) Treatment with [redacted] BIT produced statistically significant increases in the frequency of metaphases with aberrant chromosomes at 8 µg/mL in the absence of S-9 mix ($p < 0.001$, both including and excluding gap-type aberrations). These values exceeded the historical solvent control range.
4.1.2	with metabolic activation	No First Cytogenic Test (1 to 32 µg/mL [redacted] BIT) Statistically significant increases were observed in cultures treated in the presence of S-9 mix at 16 µg/mL at the first (21 hours) harvest time ($0.05 > p > 0.01$, excluding gaps only) and at 24 µg/mL at the second (45 hours) harvest time ($p < 0.001$, including gaps and $0.01 > p > 0.001$, excluding gaps). These increases did not, however, exceed the historical solvent control range (with S-9 mix: 0-9.3% including gaps and 0-5.3% excluding gaps). Second Cytogenic Test (2 to 16 µg/mL [redacted] BIT) Statistically significant increases were observed in cultures treated in the presence of S-9 mix at 16 µg/mL at harvest time (21 hours) ($0.05 > p > 0.01$, both including and excluding gaps). These values were, however, within the historical solvent control range.

Section A6 Toxicological and Metabolic Studies

Subsection A6.6.2/1 *In vitro* cytogenicity study in mammalian cells

Annex Point IIA VI.6.6.2 ***In Vitro*** **Mammalian Chromosome Aberration Test (Human Peripheral Lymphocytes).**

4.2	**Cytotoxicity**	Yes Absence of metabolic activation: Significant cytotoxicity was observed at 32 µg/mL in the main tests Presence of metabolic activation: Significant cytotoxicity was observed at 40 µg/mL in the preliminary tests (there was evidence of significant cytotoxicity in one replicate at 32 µg/mL in the first main test at the 45 hour sampling time)

5 APPLICANT'S SUMMARY AND CONCLUSION

Section A6 Toxicological and Metabolic Studies

Subsection A6.6.2/1 *In vitro* cytogenicity study in mammalian cells

Annex Point IIA VI.6.6.2 ***In Vitro*** **Mammalian Chromosome Aberration Test (Human Peripheral Lymphocytes).**

5.1 Materials and methods

The effects on chromosomal structure of exposure to [redacted] BIT were investigated in cultured human lymphocytes. Tests were conducted with and without the inclusion of a rat liver-derived metabolic activation system (S-9 mix). In the absence of metabolic activation, cells were exposed continuously for 21 and/or 45 hours; with metabolic activation, exposure was limited to three hours and cells were harvested 18 or 42 hours later (again resulting in sampling times of 21 or 45 hours).

Treatments were established by the addition of test solutions (in dimethyl sulphoxide; DMSO) to 48-hour cultures established from whole, human blood. Cell division was arrested by the addition of the spindle poison, colcemid three hours before the cells were harvested and slides were then prepared for microscopic analysis.

Mitotic indices were calculated for each culture, these were based on the number of metaphases observed per 1000 cells scored. Chromosome aberrations were scored by examination of 100 metaphases per culture and the frequencies of cells with one or more aberrations were calculated both including and excluding gap-type aberrations.

A preliminary test was performed to investigate the toxicity of [redacted] BIT over the concentration range of 8 to 5000 µg/mL to dividing lymphocytes. Exposure to [redacted] BIT in the absence and presence of metabolic activation induced significant cytotoxicity at 40 µg/mL. Subsequently the first cytogenetic test was performed using [redacted] BIT concentrations in the range 1 to 32 µg/mL, to cover the appropriate range of toxicity.

After consideration of results from the first cytogenetic test, the [redacted] BIT concentrations tested in the second cytogenetic test, at the 21 hour sampling time only, were in the range 2 to 16 µg/mL.

The main tests also incorporated solvent (DMSO) and positive (cyclophosphamide and chlorambucil) control cultures. Cyclophosphamide is a known clastogen requiring biotransformation to achieve optimum activity; chlorambucil is a direct-acting clastogen. All control and test exposures were established in duplicate cultures.

5.2 Results and discussion

Exposure to [redacted] BIT in the absence of metabolic activation induced significant cytotoxicity at 32 µg/mL (observed in all of the main toxicity tests). In the presence of metabolic activation, significant cytotoxicity was observed at 40 µg/mL [redacted] BIT in the preliminary tests. There was evidence of significant cytotoxicity in one replicate at 24 µg/mL and one replicate at 32 µg/mL in one of the main toxicity tests after the 45 hour sampling time.

In the absence of metabolic activation, treatment with [redacted] BIT at the highest concentrations selected for chromosomal analysis produced statistically significant increases in the frequency of

		metaphases with aberrant chromosomes, compared to solvent control values ($p < 0.001$, both including and excluding gap-type aberrations) at the 21 hour sampling time. The increases exceeded the historical solvent control range at the testing laboratory and were reproducible. Statistically significant increases were also observed in cultures treated in the presence of S-9 mix, but the frequencies of aberrant metaphases did not exceed the historical solvent control range. The known clastogens, cyclophosphamide and chlorambucil, induced significant increases in the frequency of metaphases with aberrant chromosomes, compared to the solvent control values, in both cytogenetic tests ($p < 0.001$ in all cases), thus demonstrating the sensitivity of the test procedure, and the metabolic activity of the S-9 mix employed. It is concluded that [redacted] BIT, under the conditions of test, was clastogenic in the absence of S-9 mix at concentrations showing moderate levels of toxicity.	
5.3	**Conclusion**	It is concluded that [redacted] BIT, under the conditions of test, was clastogenic in the absence of S-9 mix at concentrations showing moderate levels of toxicity.	
5.3.1	Reliability	1	**X**
5.3.2	Deficiencies	The study can be considered to be compatible with EC B.10 (2000) and OECD 473 (1997) with a minor discrepancy. The chemical used for the positive control (in the absence of metabolic activation) was chlorambucil which is not given as an example of a recommended positive control chemical in the guideline. Since chlorambucil induced significant increases in the frequency of metaphases with aberrant chromosomes, compared to the solvent control values, this discrepancy is not considered to have an impact on the validity of the study.	

Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*July 2021*
Materials and Methods	*Applicant's version is accepted.*
Results and discussion	*Applicant's version is accepted*

Section A6 Toxicological and Metabolic Studies

Subsection A6.6.2/1 *In vitro* cytogenicity study in mammalian cells

Annex Point IIA VI.6.6.2 ***In Vitro*** **Mammalian Chromosome Aberration Test (Human Peripheral Lymphocytes).**

Conclusion	*Applicant version is adopted*
Reliability	*2 (chlorambucil acts specifically on lymphocytes, so it is not a fully adequate positive control)*
Acceptability	*Acceptable*
Remarks	

Table A6_6_2-1: Specific Types of Aberrations- First Main Test

Absence of S-9 Mix (21 hour Sampling Time)

Treatment (µg/mL)	Culture Number	Number of Specific Types of Aberrations											
		Gaps		Breaks		Fragmentation		Exchange		Multiple Aberrations	Metaphase Aberrations		
		ctg	csg	ctb	csb	ctf	csf	cte	cse	(>8)	pul	P	E
DMSO (-)	1	1											
	2												
[redacted] BIT (4)	7	1		1								1	
	8	1		1									
[redacted] BIT (8)	9	4		6			3	4				2	
	10	3		2				2				1	
[redacted] BIT (16)	11	22		46			5	1				1	
	12	16		38		1	4	2		2		1	
CBC (2)	15	2		9			1	1				1	
	16	6		17								1	

Presence of S-9 Mix (21 hour Sampling Time)

Treatment µg/mL	Culture Number	Number of Specific Types of Aberrations											
		Gaps		Breaks		Fragmentation		Exchange		Multiple Aberrations	Metaphase Aberrations		
		ctg	csg	ctb	csb	ctf	csf	cte	cse	(> 8)	pul	P	E
DMSO (-)	17	1											
	18												
[redacted] BIT (4)	23	1		1								1	
	24			2								2	
[redacted] BIT (8)	25											1	
	26	1											
[redacted] BIT (16)	27											3	
	28	2		3				1		1		1	
CPH (6)	31	2		12			2	2					
	32	5		3			1						

CPH: Cyclophosphamide CBC: Chlorambucil
Ctg: Chromatid gap csg: Chromosome gap
ctb: Chromatid break csb: Chromosome break
ctf: Chromatid fragment csf: Chromosome fragment
cte: Chromatid exchange cse: Chromosome exchange
Pul: Pulverised metaphase P: Polyploid metaphase
E: Endoreduplicated metaphase
pul, P and E are not included in the total number of cells scored for aberrations.
Where no number is given, no aberrations of that type were observed for that culture.

Table A6_6_2-2: Specific Types of Aberrations- First Main Test (repeat)

Presence of S-9 Mix (45 hour Sampling Time)

Treatment (µg/mL)	Culture Number	Number of Specific Types of Aberrations											
		Gaps		Breaks		Fragmentation		Exchange		Multiple Aberrations	Metaphase Aberrations		
		ctg	csg	ctb	csb	ctf	csf	cte	cse	(> 8)	pul	P	E
DMSO (-)	47	1											
	48	2											
[redacted] BIT (24)	49	5		6				1		1		2	1
	50	5		3			1					1	1
CPH (12)	55	4		7		1	3						1
	56	2		10			2	1					

CPH: Cyclophosphamide Ctg: Chromatid gap
csg: Chromosome gap ctb: Chromatid break
csb: Chromosome break ctf: Chromatid fragment
csf: Chromosome fragment cte: Chromatid exchange
cse: Chromosome exchange Pul: Pulverised metaphase
P: Polyploid metaphase E: Endoreduplicated metaphase
pul, P and E are not included in the total number of cells scored for aberrations.
Where no number is given, no aberrations of that type were observed for that culture.

Table A6_6_2-3: Specific Types of Aberrations- Second Main Test

Absence of S-9 Mix (21 hour Sampling Time)

Treatment µg/mL	Culture Number	Number of Specific Types of Aberrations											
		Gaps		Breaks		Fragmentation		Exchange		Multiple Aberrations (> 8)	Metaphase Aberrations		
		ctg	csg	ctb	csb	ctf	csf	cte	cse		pul	P	E
DMSO (-)	1											1	
	2			2								1	
[redacted] BIT (2)	3			1									
	4	1		1								1	
[redacted] BIT (4)	5	1		1									
	6	1		1									
[redacted] BIT (8)	7	5		12								1	
	8	5		6				3					
CBC	11	25	1	65			7	17		3			
	12	25		57		1	2	22					

Presence of S-9 Mix (21 hour Sampling Time)

Treatment (µg/mL)	Culture Number	Number of Specific Types of Aberrations											
		Gaps		Breaks		Fragmentation		Exchange		Multiple Aberrations (>8)	Metaphase Aberrations		
		ctg	csg	ctb	csb	ctf	csf	cte	cse		pul	P	E
DMSO (-)	13	1										3	
	14			1									
[redacted] BIT (4)	17	2		3			1					1	
	18			1									
[redacted] BIT (8)	19	1		5								1	
	20	1										1	
[redacted] BIT (16)	21	2		2				1					
	22			5			1	1				3	
CPH (6)	23	8		32			1	7					
	24	3		18		1	1	4				1	

CPH: Cyclophosphamide CBC: Chlorambucil
Ctg: Chromatid gap csg: Chromosome gap
ctb: Chromatid break csb: Chromosome break
ctf: Chromatid fragment csf: Chromosome fragment
cte: Chromatid exchange cse: Chromosome exchange
Pul: Pulverised metaphase P: Polyploid metaphase
E: Endoreduplicated metaphase
pul, P and E are not included in the total number of cells scored for aberrations.
Where no number is given, no aberrations of that type were observed for that culture.

Table A6_6_2-4: Summary of Reduction in Mitotic Index and Chromosomal Aberrations Observed in the Main Cytogenic Tests

Treatment (µg/mL)	Reduction in Mean MI (%)	Cells with aberrations-Including gaps			Cells with aberrations-Excluding gaps		
		Individual Values (%)	Mean (%)	S.S.	Individual Values (%)	Mean (%)	S.S.
First Cytogenic Test							
Absence of S-9 mix - 21 hour sampling time							
DMSO (-)	-	1, 0	0.5	-	0, 0	0.0	-
BIT (4)	6	2, 2	2.0	NS	1, 1	1.0	NS
BIT (8)	46	11, 7	9.0	***	8, 4	6.0	***
BIT (16)	56	35, 37	36.0	***	25, 29	27.0	***
CBC (2)	4	11, 20	15.5	***	9, 15	12.0	***
Presence of S-9 mix - 21 hour sampling time							
DMSO (-)	-	1, 0	0.5	-	0, 0	0.0	-
BIT (4)	30	2, 2	2.0	NS	1, 2	1.5	NS
BIT (8)	19	0, 1	0.5	NS	0, 0	0.0	NS[a]
BIT (16)	35	0, 6	3.0	NS	0, 5	2.5	*
CPH (6)	70	18, 9	13.5	***	16, 4	10.0	***
Presence of S-9 mix - 45 hour sampling time							
DMSO (-)	-	1, 2	1.5	-	0, 0	0.0	-
BIT (24)	55	9, 9	9.0	***	4, 4	4.0	**
CPH (6)	0	15, 15	15.0	***	11,13	12.0	***
Second Cytogenic Test							
Absence of S-9 mix - 21 hour sampling time							
DMSO (-)	-	0, 2	1.0	-	0, 2	1.0	-
BIT (2)	Increase	1, 2	1.5	NS	1, 1	1.0	NS
BIT (4)	17	2, 2	2.0	NS	1, 1	1.0	NS

Treatment (µg/mL)	Reduction in Mean MI (%)	Cells with aberrations-Including gaps			Cells with aberrations-Excluding gaps		
		Individual Values (%)	Mean (%)	S.S.	Individual Values (%)	Mean (%)	S.S.
BIT (8)	42	15, 11	13.0	***	10, 7	8.5	***
CBC (2)	52	63, 51	57.0	***	54, 42	48.0	***
Presence of S-9 mix - 21 hour sampling time							
DMSO (-)	-	1, 1	1.0	-	0, 1	0.5	-
BIT (4)	7	6, 1	3.5	NS	4, 1	2.5	NS
BIT (8)	10	5, 1	3.0	NS	4, 0	2.0	NS
BIT (16)	63	5, 4	4.5	*	3, 4	3.5	*
CPH (6)	54	34, 22	28.0	***	28, 20	24.0	***

Reduction in mean MI (%): Reduction in mean mitotic index compared to negative control values
S.S: Statistical significance of increase in frequency of aberrant metaphases in treated cultures, compared to negative controls
NS[a]: Not significant, zero aberrant metaphases in both control and treated groups
NS: Not significant, $p > 0.05$ *: Significant, $0.05 > p > 0.01$
: Highly significant, $0.01 > p > 0.001$ *: Very highly significant, $p < 0.001$
CBC: Chlorambucil CPH: Cyclophosphamide
BIT: [redacted] BIT

Section A6 Toxicological and Metabolic Studies

Subsection A6.6.3/1 *IN VITRO* GENE MUTATION ASSAY IN MAMMALIAN CELLS (L5178Y MOUSE LYMPHOMA CELLS)

Annex Point IIA VI.6.6.3

Mouse Lymphoma Cells

			Official use only
		1 REFERENCE	
1.1	**Reference**	[redacted] 2003; BIT: Mutation at the *hprt* locus of L5178Y Mouse Lymphoma Cells using the Microtitre Fluctuation Technique. [redacted] Report No. 2075/7-D6173	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Clariant Production UK Ltd	
1.2.2	Company with letter of access	Arch Chemicals Inc and Thor GmbH	
1.2.3	Criteria for data protection	Data on existing substance for first entry into Annex I.	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	OECD 476 (1997), EC B.17 (2000)	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	[redacted]	
3.1.2	Specification	As given in section 2	
3.1.2.1	Purity	Purity: 77.4% 1,2-Benzisothiazol-3-(2*H*)-one (BIT) All test article concentrations were expressed in terms of the active ingredient BIT, taking the 22.5% moisture content of the sample into account	
3.1.2.2	Stability	Stable under storage conditions of ambient temperature in the dark	
3.2	**Study Type**	*In vitro* mammalian cell gene mutation test	
3.2.1	Organism/cell type	Mouse lymphoma L5178Y cells	
3.2.2	Deficiencies / Proficiencies	Hypoxanthine-guanine PhosphoRibosyl Transferase (HPRT) proficient/deficient (6-thioguanine (6TG) resistance selects cells	

		deficient in HPRT)
3.2.3	Metabolic activation system	S9 mix, rat, liver, induced, Aroclor 1254
3.2.4	Positive control	4-nitroquinoline 1-oxide (NQO), -S9 Benzo(a)pyrene (BP), +S9
3.3	**Application of test substance**	
3.3.1	Concentrations	<u>Cytotoxicity Range Finding Experiment 1</u> 46.88 to 1512 µg/mL (with and without metabolic activation) <u>Cytotoxicity Range Finding Experiment 2</u> 0.3906, 0.7813, 1.563, 3.125, 6.25, 12.5, 25 and 50 µg/mL (with and without metabolic activation) <u>Experiment 1</u> 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1.2, 1.8, 2.4 and 3.2 µg/mL (without metabolic activation) 0.2, 0.4, 0.8, 1.2, 1.6, 2.4, 3.2 and 6.4 µg/mL (with metabolic activation) <u>Experiment 2</u> 0.8, 1.2, 1.6, 2.4, 3.6, 4.8 and 6.4 µg/mL (without metabolic activation) 1.6, 2.4, 3.2, 4.8, 6.4, 9.6 and 12.8 µg/mL (with metabolic activation)
3.3.2	Way of application	Dissolved in medium
3.3.3	Pre-incubation time	Not applicable
3.3.4	Other modifications	Not applicable
3.4	**Examinations**	
3.4.1	Number of cells evaluated	Results expressed as number of induced mutants per 10^6 survivors.
		4 RESULTS AND DISCUSSION
4.1	**Genotoxicity**	
4.1.1	without metabolic	No

	activation	
4.1.2	with metabolic activation	A small but statistically significant increase in mutant frequency was observed at the highest dose analysed in Experiment 2 (6.4 µg/mL), compared to the concurrent solvent controls, and a linear trend was observed. The mutant frequency observed at this dose was, however, similar to the historical mean solvent control mutant frequency. Furthermore, the statistically significant increase in mutant frequency was observed at a highly toxic dose, yielding only 10% relative survival. This increase in mutant frequency was therefore considered of little or no biological significance.
4.2	**Cytotoxicity**	<u>Cytotoxicity Range Finding Experiment 2</u> Cytotoxic (< 10% relative survival) at the following concentrations: Without metabolic activation: 50 to 1.563 µg/mL With metabolic activation: 50 to 3.125 µg/mL <u>Experiment 1</u> Without metabolic activation: > 3.2 µg/mL (61% relative survival at 3.2 µg/mL which was the highest test concentration) With metabolic activation: > 3.2 µg/mL (44% relative survival at 3.2 µg/mL; there was extreme toxicity observed in one of the replicate cultures at 4.8 µg/mL; the 6.4 µg/mL dose level could not be analysed since the plates were contaminated) <u>Experiment 2</u> Without metabolic activation: 6.4 µg/mL (3% relative survival was observed at 6.4 µg/mL; there was 9% relative survival observed at 4.8 µg/mL which was considered to be an acceptable maximum level of toxicity) With metabolic activation: 9.6 µg/mL (3% relative survival at 9.6 µg/mL; there was 10% relative survival observed at 6.4 µg/mL)
	5 APPLICANT'S SUMMARY AND CONCLUSION	
5.1	**Materials and methods**	BIT was assayed for mutation at the *hprt* locus (6-thioguanine resistance) in mouse lymphoma cells using a fluctuation protocol (guideline compliance with OECD 476 (1997) and EC B.17 (2000)). The study consisted of cytotoxicity range-finding experiments followed by two independent experiments, each conducted in the absence and presence of metabolic activation by an Aroclor 1254 induced rat liver post-mitochondrial fraction (S-9). The initial cytotoxicity range-finding experiment with and without metabolic activation was performed in the concentration range 46.88 to 1512 µg/mL. Since complete toxicity was observed at all

concentrations, a second range-finding experiment was performed with and without metabolic activation in the range 0.3906 to 50 µg/mL. On the basis of the results from this second range-finding experiment, Experiment 1 of the main test was performed in the concentration range 0.1 to 3.2 µg/mL in the absence of metabolic activation and in the concentration range 0.2 to 6.4 µg/mL in the presence of metabolic activation. The concentration range was extended in Experiment 2 of the main test to 0.2 to 6.4 µg/mL in the absence of metabolic activation and to 0.4 to 12.8 µg/mL in the presence of metabolic activation.

Cultures with BIT, 4-Nitroquinoline-1-oxide (NQO, positive control in the absence of metabolic activation) or Benzo(a)pyrene (BP, positive control in the presence of metabolic activation) were maintained in flasks for a period of 7 days during which the HPRT mutation would be expressed.

Mutant frequency was assessed for statistical significance. The experiment was considered valid if the mutant frequencies in the solvent control cultures fell within the normal range (not more than three times the historical mean value) and at least one concentration of each of the positive control chemicals induced a clear increase in mutant frequency (the difference between the mutant frequencies was greater than half the historical mean value).

The test substance was considered to be mutagenic if the assay was valid, the mutant frequency of one or more doses was significantly greater than that of the solvent control, and there was a significant dose relationship as indicated by the linear trend analysis and if these effects were reproducible.

5.2 Results and discussion

In Experiment 1, the highest dose selected in the presence of S-9 (6.4 µg/mL) could not be analysed due to excessive contamination observed on the mutant plates and the second highest dose tested (4.8 µg/mL) was considered too toxic for selection to determine viability and 6-thioguanine (6TG) resistance (extreme toxicity in one of the replicate cultures was observed). The highest dose analysed in both the absence and presence of S-9 was therefore 3.2 µg/mL, with relative survival being 61% and 44%, respectively. No dose of ideal toxicity (10-20% relative survival) was achieved in the absence or presence of S-9. This was unexpected, based on the results of the cytotoxicity range-finding experiment. However, adequate, dose-related toxicity was demonstrated in the absence and presence of S-9 in Experiment 2, therefore this was not considered to have affected the integrity of the study in any way.

In Experiment 2, the highest doses analysed were 4.8 µg/mL in the absence of S-9 and 6.4 µg/mL in the presence of S-9, with relative survival being 9% and 10%, respectively.

Negative (solvent) and positive control treatments were included in each mutation experiment in the absence and presence of metabolic

		activation. Mutant frequencies in negative control cultures fell within normal ranges, and clear increases in mutation were induced by the positive control chemicals 4-Nitroquinoline-1-oxide (without metabolic activation) and Benzo(a)pyrene (with metabolic activation). The study was therefore considered valid. No statistically significant increases in mutant frequency were observed following treatment with BIT at any dose level analysed, in the absence or presence of metabolic activation in Experiment 1, or in the absence of metabolic activation in Experiment 2. A small but statistically significant increase in mutant frequency was observed at the highest dose analysed in the presence of metabolic activation in Experiment 2 (6.4 µg/mL), compared to the concurrent solvent controls, and a linear trend was observed. The mutant frequency observed at this dose was, however, similar to the historical mean solvent control mutant frequency. Furthermore, the statistically significant increase in mutant frequency was observed at a highly toxic dose, yielding only 10% relative survival. This increase in mutant frequency was therefore considered of little or no biological significance.
5.3	**Conclusion**	Under the conditions employed in this study, BIT did not show conclusive evidence of mutagenic activity.
5.3.1	Reliability	1
5.3.2	Deficiencies	No

Evaluation by Competent Authorities

EVALUATION BY RAPPORTEUR MEMBER STATE

Date	*September 2008*
Materials and Methods	*Applicant's version is accepted.*
Results and discussion	*Applicant's version is accepted.*
Conclusion	*Applicant's version is adopted.*
Reliability	*1*
Acceptability	*Acceptable*

Section A6 Toxicological and Metabolic Studies

Subsection A6.6.3/1

Annex Point IIA VI.6.6.3

IN VITRO GENE MUTATION ASSAY IN MAMMALIAN CELLS (L5178Y MOUSE LYMPHOMA CELLS)

Mouse Lymphoma Cells

Remarks

Table A6_6_3-1: Summary of Cell Viability and 6-Thioguanine Resistance (Experiment 1)

Treatment (µg/mL)	-S9			Treatment (µg/mL)	+S9		
	% RS[a]	MF[b]			% RS[a]	MF[b]	
BIT							
0	100.00	7.59	-	0[c]	100.00	15.47	-
0.1	77.34	9.80	NS	0.2	43.56	16.03	NS
0.2	101.71	15.50	NS	0.4[c]	62.87	10.84	NS
0.3	86.72	14.75	NS	0.8	88.67	13.24	NS
0.4	52.73	15.79	NS	1.2	61.18	10.96	NS
0.6	58.99	15.74	NS	1.6	81.24	11.73	NS
0.8	67.82	12.97	NS	2.4	67.88	12.81	NS
1.2	118.91	8.97	NS	3.2	44.34	15.02[f]	NS
1.8	77.99	13.47	NS	4.8[d]	40.09	-	-
2.4	80.80	12.03	NS	6.4[e]	29.97	-	-
3.2	60.66	14.77	NS	-	-	-	-
Linear Trend: NS				Linear Trend: NS			
4-Nitroquinoline-1-oxide				Benzo(a)pyrene			
0.1	63.71	32.66		2	43.49	46.48	
0.15	106.00	38.57		3	31.75	70.35	

-S9: Without metabolic activation
+S9: With metabolic activation
a: %RS Percent relative survival adjusted by post treatment cell counts
b: 6-TG resistant mutants/10^6 viable cells 7 days after treatment
c: Treatment has high heterogeneity, but is included in analysis
d: Not plated for viability (6-TG resistance)
e: Mutation plates were not scored due to contamination
f: Based on one replicate only
NS: Not Significant

Table A6_6_3-2: Summary of Cell Viability and 6-Thioguanine Resistance (Experiment 2)

Treatment (µg/mL)	-S9			Treatment (µg/mL)	+S9		
	% RS[a]	MF[b]			% RS[a]	MF[b]	
BIT							
0	100.00	3.30	-	0	100.00	3.36	-
0.2 [c]	83.37	-	-	0.4[c]	72.97	-	-
0.4 [c]	67.74	-	-	0.8 [c]	60.13	-	-
0.6 [c]	54.40	-	-	1.6	47.18	3.11	NS
0.8	47.81	4.21	NS	2.4	42.20	3.00	NS
1.2	41.28	3.57	NS	3.2	25.64	4.06	NS
1.6	41.07	3.16	NS	4.8	21.00	6.18	NS
2.4	31.54	5.07	NS	6.4	10.33	9.90	*[e]
3.6	21.27	5.28	NS	9.6[d]	3.14	20.88	-
4.8	9.40	9.09	NS	12.8[d]	0.16	13.26	-
6.4 [d]	2.87	4.28	-	-	-	-	-
Linear Trend: *[e]				Linear Trend: ***[f]			
4-Nitroquinoline-1-oxide				Benzo(a)pyrene			
0.1	63.38	35.10		2	72.12	90.83	
0.15	65.61	44.29		3	53.85	84.58	

-S9: Without metabolic activation
+S9: With metabolic activation
a: %RS Percent relative survival adjusted by post treatment cell counts
b: 6-TG resistant mutants/10^6 viable cells 7 days after treatment
c: Not plated for viability (6-TG resistance)
d: Treatment excluded from final test statistics due to excessive toxicity
e: Comparison of each treatment with control: Dunnetts test (one-sided), significant at 5% level
f: Test for linear trend: x^2(one-sided), significant at 5%, 1% and 0.1% level respectively
NS: Not Significant

Section A6 Toxicological and Metabolic Studies

Subsection A6.6.4 Genotoxicity *In Vivo* micronucleus assay

Annex Point IIA VI.6.6.4

			Official use only
		1 REFERENCE	
1.1	**Reference**	[redacted] 1995; [redacted] BIT: Mouse Micronucleus Test to Comply with O.E.C.D. Guideline 474 (1983). [redacted]., Report No. 95/NLL057/0765	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Clariant Production UK Ltd	
1.2.2	Company with letter of access	Arch Chemicals Inc, Thor GmbH	
1.2.3	Criteria for data protection	Data on existing substance for first entry in to Annex I.	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	OECD 474 (1983), EC B.12 (1992) Considered largely compatible with OECD 474 (1997), EC B.12 (2000)	
2.2	**GLP**	Yes	
2.3	**Deviations**	See 3.2.7 and 5.3.2	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	[redacted]	
3.1.2	Specification	As given in section 2	
3.1.2.1	Purity	The [redacted] BIT used in this study had a reported organic purity of 99.8% of which 98.8% is BIT.	
3.1.2.2	Stability	Stable under storage conditions of ambient temperature in the dark.	
3.1.2.3	Maximum tolerable dose	1200 mg [redacted] BIT/kg body weight	
3.2	**Test Animals**	Non-entry field	
3.2.1	Species	Mouse	
3.2.2	Strain	CD-1	

Section A6 Toxicological and Metabolic Studies

Subsection A6.6.4 Genotoxicity *In Vivo* micronucleus assay

Annex Point IIA VI.6.6.4

3.2.3	Source	[redacted]
3.2.4	Sex	Male and Female
3.2.5	Age/weight at study initiation	The animals were 4-5 weeks old when received from the breeding unit. The animals were acclimatised for at least 4 days prior to treatment.
3.2.6	Number of animals per group	Preliminary Toxicity Test 2 male and 2 female at each dose group of 125, 250, 500, 1000, 2000 and 5000 mg/kg. Main Micronucleus Test 5 male and 5 female per group (300 and 600 mg/kg [redacted] BIT and chlorambucil control group). 15 male and 15 female per group (corn oil control group and 1200 mg/kg [redacted] BIT Group). Animals were randomly allocated to sex groups for the preliminary and main test. Animal Husbandry and Environmental Control Drinking water and Laboratory animal diet RM1(E)SQC (manufactured by Special Diets Services Ltd., Witham, Essex, UK) was fed *ad libitum* throughout the study. This diet contains no added antibiotic or other chemotherapeutic or prophylactic agent. The animals were housed inside a barriered, limited-access rodent facility. Temperature and humidity controls were designed to maintain conditions in the ranges 19-23°C and 45-60 % relative humidity. There were approximately 15 air changes per hour and a 12 hour light/12hour dark cycle in operation.
3.2.7	Control animals	Vehicle Control: Corn Oil Positive Control: Chlorambucil
3.3	**Administration/ Exposure**	Oral
3.3.1	Number of applications	One
3.3.2	Interval between applications	Not applicable

Section A6 Toxicological and Metabolic Studies

Subsection A6.6.4 Genotoxicity *In Vivo* micronucleus assay

Annex Point IIA VI.6.6.4

3.3.3	Postexposure period	Preliminary Toxicity Test Post exposure period: 72 hours (all animals were sacrificed 72 hours after treatment). Main Micronucleus Test Post exposure period: 24, 48 and 72 hours as detailed below: 24 hours after treatment 5 males and 5 females from each treatment group were sacrificed. 48 hours after treatment a further 5 males and 5 females from the corn oil control group and 1200 mg/kg [redacted] BIT Group were sacrificed. 72 hours after treatment the remaining 5 males and 5 females from the corn oil control group and 1200 mg/kg [redacted] BIT Group were sacrificed.
3.3.4	Type	Gavage

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Subsection A6.6.4 Genotoxicity *In Vivo* micronucleus assay

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3.3.5 Concentration

Preliminary Toxicity Test

Group	Treatment	Dosage (mg/kg body weight)	Number of Mice
1	[redacted] BIT	125	2 M, 2 F
2	[redacted] BIT	250	2 M, 2 F
3	[redacted] BIT	500	2 M, 2 F
4	[redacted] BIT	1000	2 M, 2 F
5	[redacted] BIT	2000	2 M, 2 F
6	[redacted] BIT	5000	2 M, 2 F

Main Micronucleus Test

Group	Treatment	Dosage (mg/kg body weight)	Number of Mice
1	Corn Oil	-	15 M, 15 F
2	[redacted] BIT	300	5 M, 5 F
3	[redacted] BIT	600	5 M, 5 F
4	[redacted] BIT	1200	15 M, 15 F
6	Chlorambucil	30	5 M, 5 F

3.3.6 Vehicle

Corn oil

3.3.7 Concentration in vehicle

The concentrations of the dosing solutions were not reported.

Dosing solutions were prepared freshly in corn oil on the day of dosing, each concentration (based on individual body weights) being individually formulated and mixed prior to use.

The test material was found to form a doseable and apparently homogeneous (no determinations of homogeneity were performed) suspension in corn oil at a maximum concentration of approximately 500 mg/mL (maximum concentration of dosing solution calculated as approximately 120 mg/mL).

No determinations of stability or concentration were made, however the dosing solutions were prepared on the day of dosing.

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Subsection A6.6.4 Genotoxicity *In Vivo* micronucleus assay

Annex Point IIA VI.6.6.4

3.3.8	Total volume applied	10 mL dosing solution/kg body weight
3.3.9	Controls	Vehicle Control: 10 mL corn oil/kg body weight. Positive Control: Chlorambucil, administered once orally at a dosage of 30 mg/kg in aqueous 10% ethanol.
3.4	**Examinations**	
3.4.1	Clinical signs	Clinical observations were made during the acclimatisation period, preliminary test and main micronucleus test. Animals were inspected daily for signs of ill-health or reaction to treatment. Any deviation from normal was recorded. Body weights were recorded for all animals on the day of treatment and again immediately before termination, and bodyweights were recorded. In addition, the animals in the preliminary toxicity test were weighed immediately prior to dosing and daily thereafter until termination.
3.4.2	Tissue	Bone marrow. Animals were killed by cervical dislocation following carbon dioxide inhalation. Femurs of each animal were rapidly dissected and cleaned out of adherent tissue. Marrow cells were flushed out with foetal calf serum, the recovered cells were centrifuged and the majority of the supernatant fluid was discarded. Single drops of the cell suspension were transferred to clean, dry slides, two or three smears (for the preliminary toxicity test or main micronucleus test respectively) prepared, and the slides left to air-dry. Following fixation in methanol for ten minutes, they were stained manually, using 5% Giemsa stain. After staining, slides were washed in buffer, allowed to air-dry, cleared in xylene, and made permanent using DPX mountant.
	Number of animals:	Slides were examined from all animals.

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Number of cells:	Preliminary Test A minimum of 1000 cells from each animal were scored (from a minimum of 2000 erythrocytes examined). Each erythrocyte was classed as polychromatic or mature. The slides were examined under the light microscope, and regions judged to be of adequate technical quality to permit scoring were selected under low magnification. At high magnification (x 1000, oil immersion) a total of at least 2000 erythrocytes per animal were examined. Each erythrocyte scored was classed as polychromatic or mature: polychromatic cells stain blue/pink and the older cells stain red/pink. At least 1000 cells of each type were scored from each animal where possible, but where there was an appreciable deviation from unity in the ratio of polychromatic to mature erythrocytes, scoring continued until a minimum of 2000 of the predominant cell type were counted. Main Micronucleus Test A minimum of 1000 cells from each animal were scored (from a minimum of 2000 erythrocytes examined). Each erythrocyte was classed as polychromatic or mature and examined for the absence or presence of micronuclei. At least one slide from each animal was randomly coded and care was taken to ensure that no unique slide identifications remained visible in order to eliminate bias. Slides were examined as detailed for the preliminary toxicity test, but in addition each erythrocyte scored was examined for the presence or absence of micronuclei. The frequencies of micronucleated cells per 1000 erythrocytes were calculated. The incidence of micronuclei in the mature cell population 24 hours after treatment reflects the pretreatment situation, since most of these cells were produced before treatment. The frequency of micronuclei in polychromatic cells provides an index of induced genetic damage. The ratio of polychromatic to mature cells was also determined; a decrease in this may indicate inhibition of cell division following treatment.

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Subsection A6.6.4 Genotoxicity *In Vivo* micronucleus assay

Annex Point IIA VI.6.6.4

	Time points:	Preliminary Toxicity Test 72 hours after treatment. Main Micronucleus Test 24 Hours after treatment: 5 males and 5 females from each treatment group. 48 hours after treatment: Further 5 males and 5 females from corn oil control group and 1200 mg/kg [redacted] BIT Group. 72 hours after treatment: Remaining 5 males and 5 females from corn oil control group and 1200 mg/kg [redacted] BIT Group.
	Type of cells	Erythrocytes in bone marrow.
	Parameters:	Polychromatic/normochromatic erythrocyte ratio. Frequency of micronucleated cells.
3.5	**Further remarks**	Statistical Analysis of Results The frequencies of micronucleated cells per 1000 polychromatic erythrocytes scored were subjected to statistical analysis by the Mann-Whitney U procedure (Mann and Whitney, 1942). A computer software version of this test was employed and significance was determined by reference to tabulated values of R_1. Data from males and females within each group were compared using a two-tailed test. Where there was no significant difference within the group, the sexes were pooled for further analysis. For each sampling time (24, 48 or 72 hours), each treated group was compared with concurrent vehicle controls using a one-tailed test.
		4 RESULTS AND DISCUSSION
4.1	**Clinical signs**	**Preliminary Toxicity Test** Mice dosed at 5000 mg/kg showed adverse reactions to treatment including hunched posture (4 animals), underactivity (3), piloerection (3), partially closed eyes (3), prostrate posture (2), slow respiration (2), skin pallor (2), noisy respiration (1), bradypnoea (1), low body temperature (1) and slight abdominal distension (1). One male was found dead approximately 3 hours after dosing and one female was killed *in extremis* approximately 24 hours after dosing. Mice treated at 2000 mg/kg showed adverse reactions to treatment including hunched posture (4 animals), piloerection (3), partially closed eyes (3), noisy respiration (3), underactivity (2), prostrate posture (1), post dose salivation (1), skin pallor (1) and vocalisation (1). One male was killed *in extremis* approximately 24 hours after dosing. One male dosed at 1000 mg/kg showed hunched posture,

piloerection, noisy respiration and distended abdomen; the other male showed transient hunched posture after dosing. No adverse reactions to treatment were observed in the two females.

One male dosed at 500 mg/kg showed hunched posture, piloerection and noisy respiration after dosing. No adverse reactions to treatment were observed in the other male or either female.

No adverse reactions to treatment were observed in mice dosed at 125 or 250 mg/kg.

There was some evidence of increased weight loss in mice dosed at 1000 mg/kg and above (body weight at termination was compared to Day 1, no statistical analysis was performed).

Main Micronucleus Test

After dosing at 1200 mg/kg, all thirty animals showed adverse reactions to treatment including hunched posture (30 animals), piloerection (28), underactivity (14), noisy respiration (7), partially closed eyes (5), prostrate posture (2), distended abdomen (1), abnormal gait (1) and skin pallor (1); four of these mice were found dead between 2 and 47 hours after dosing. Transient adverse reactions to treatment were also observed in two mice dosed at 600 mg/kg: one showed underactivity, hunched posture and partially closed eyes and another showed hunched posture.

Body weights were recorded at the times of dosing and termination. In view of the relatively short time interval between weighings, no major significance can be attached to the body weight data for animals dosed with [redacted] BIT and terminated after 24 hours of exposure. There was some evidence of increased body weight loss in mice dosed at 1200 mg/kg [redacted] BIT and sacrificed 48 or 72 hours after dosing.

Eight out of the ten mice dosed with the positive control agent, chlorambucil, lost weight during the 24 hour period before termination.

Section A6 Toxicological and Metabolic Studies

Subsection A6.6.4 Genotoxicity *In Vivo* micronucleus assay

Annex Point IIA VI.6.6.4

4.2	**Haematology / Tissue examination**	/	Preliminary Test Mice dosed with [redacted] BIT at 2000 and 5000 mg/kg showed reduced bone marrow proliferation (the mean ratio of polychromatic to mature erythrocytes was reduced to 0.5 and 0.4, respectively). After consideration of these data, the highest dosage of [redacted] BIT selected for the main micronucleus test was 1200 mg/kg. Main Micronucleus Test A statistically significant difference ($0.05 > p > 0.01$) in the frequency of micronucleated polychromatic erythrocytes was observed between males and females dosed with [redacted] BIT at 1200 mg/kg and killed after 24 hours. This difference is not considered to be of biological significance because the values (range 0.9-3.9 for males and 0.0-1.0 for females) were all within the historical vehicle control range at the testing laboratory (0.0-4.9) and no differences were observed between the sexes after 48 or 72 hours of treatment. The incidence of micronucleated polychromatic erythrocytes in groups treated with [redacted] BIT was similar to vehicle control group values at each sacrifice time. Statistical analysis confirmed that there was no significant difference between the vehicle control group and any group treated with [redacted] BIT, at any termination time ($p > 0.05$). Treatment with Chlorambucil produced a large, statistically significant increase in the frequency of micronucleated polychromatic erythrocytes ($p < 0.01$). This increase after exposure to a known mutagen demonstrates the sensitivity of the test system. The recorded incidence of micronuclei per 1000 mature erythrocytes varied between 0.0 and 3.4 throughout all groups. These findings demonstrate the normal status of the animals used in the study: in particular, the low incidence in animals killed 24 hours after treatment shows the absence of any pre-treatment abnormality in the bone marrow. The ratio of polychromatic to mature erythrocytes for all groups treated with [redacted] BIT were closely similar to corresponding vehicle control group values at each sacrifice time. In animals treated with chlorambucil, the ratio between polychromatic and mature erythrocytes was reduced (0.6).
4.3	**Genotoxicity**		No There was no evidence of induced chromosomal or other damage leading to micronucleus formation in polychromatic erythrocytes of treated mice 24, 48 or 72 hours after oral administration of [redacted] BIT.

Section A6 Toxicological and Metabolic Studies

Subsection A6.6.4 Genotoxicity *In Vivo* micronucleus assay

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4.4	**Other**	Not applicable

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1	**Materials and methods**	The effect of [redacted] BIT on chromosome structure in bone marrow cells was investigated following acute oral administration to mice according to OECD 474 (1983). Chromosome damage was measured indirectly by counting micronuclei. A preliminary toxicity test was conducted using dosages of 125, 250, 500, 1000, 2000 and 5000 mg/kg. Adverse reactions to treatment were observed in all mice dosed at 2000 and 5000 mg/kg, two mice dosed at 1000 mg/kg and a single mouse dosed at 500 mg/kg. Two mice dosed at 5000 mg/kg and one mouse dosed at 2000 mg/kg were found dead or killed *in extremis* within 24 hours of dosing. Subsequently, in the main micronucleus test, male and female mice were given a single dose of [redacted] BIT at 300, 600 or 1200 mg/kg. In all cases [redacted] BIT was dosed orally, suspended in corn oil. Concurrent vehicle and positive control groups of mice were similarly dosed with corn oil or chlorambucil (30 mg/kg) respectively. Five males and five females from each group were killed 24 hours after treatment; further lots of five males and five females, given [redacted] BIT at 1200 mg/kg or the vehicle control, were killed 48 and 72 hours after treatment. Bone marrow smears on glass slides were made from each animal. These slides were then stained and prepared for examination. At least 2000 erythrocytes per animal were then examined for the presence of micronuclei, using the light microscope. Calculated values of micronuclei per 1000 polychromatic erythrocytes were analysed statistically using the Mann-Whitney U test. The ratio of polychromatic:mature cells was also calculated for each group, as an indicator of gross toxicity.

Section A6 Toxicological and Metabolic Studies

Subsection A6.6.4 Genotoxicity *In Vivo* micronucleus assay

Annex Point IIA VI.6.6.4

5.2	**Results and discussion**	Adverse reactions to treatment were observed in all thirty mice dosed with [redacted] BIT at 1200 mg/kg, including hunched posture (30 mice), piloerection (28), underactivity (14), noisy respiration (7), partially closed eyes (5), prostrate posture (2), distended abdomen (1), abnormal gait (1) and skin pallor (1); four of these mice were found dead between 2 and 47 hours after dosing. Transient reactions to treatment were also observed in two mice dosed at 600 mg/kg. No real indication of bone marrow toxicity, as evidenced by depression of bone marrow proliferation, was noted in any group treated with [redacted] BIT. Frequencies of micronucleated polychromatic erythrocytes in animals killed 24, 48 or 72 hours after administration of [redacted] BIT were similar to those in concurrent vehicle controls. This lack of treatment related effect was apparent in both sexes, and was confirmed by statistical analysis. The sensitivity of the test was shown by statistically significant increases in the frequency of micronucleated polychromatic erythrocytes over control values in positive control group animals given chlorambucil at 30 mg/kg ($p < 0.01$). It is concluded that, under the conditions of test, there was no evidence of induced chromosomal or other damage leading to micronucleus formation in polychromatic erythrocytes of treated mice 24, 48 or 72 hours after oral administration of [redacted] BIT.
5.3	**Conclusion**	There was no evidence of induced chromosomal or other damage leading to micronucleus formation in polychromatic erythrocytes of treated mice 24, 48 or 72 hours after oral administration of [redacted] BIT.
5.3.1	Reliability	1
5.3.2	Deficiencies	The study can be considered to be essentially compatible with the current OECD Guideline 474 with a minor discrepancy. The positive control chemical was chlorambucil, which is not suggested in the current guideline. Treatment with chlorambucil produced a large, statistically significant increase in the frequency of micronucleated polychromatic erythrocytes ($p < 0.01$). Chlorambucil is a known mutagen and this increase in frequency of micronucleated polychromatic erythrocytes demonstrates the sensitivity of the test system. Therefore this discrepancy is not considered to affect the validity of the study.

Evaluation by Competent Authorities

Section A6 Toxicological and Metabolic Studies

Subsection A6.6.4 Genotoxicity *In Vivo* micronucleus assay

Annex Point IIA VI.6.6.4

	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*September 2008*
Materials and Methods	*Applicant's version is accepted.*
Results and discussion	*Applicant's version is accepted*
Conclusion	*Applicant's version is adopted.*
Reliability	*1*
Acceptability	*Acceptable*
Remarks	

Table 6_6_4-1: Micronucleus Test: Data Summary and Statistical Analysis

Sacrifice Time	Group	Treatment (mg/kg)		MnP cells/ 1000		S.S.		P:M
				Mean ± SD	Range	MvsF	Treated vs Control	
24 Hours	1	Vehicle (-)	M	1.0±1.7	0.0-4.0	NS	-	0.7
			F	0.8±1.1	0.0-2.0		-	0.8
			Group	0.9±1.4	0.0-4.0	-	-	0.7
	2	[redacted] BIT (300)	M	2.2±1.8	0.0-4.0	NS	-	0.7
			F	0.6±1.3	0.0-3.0		-	0.8
			Group	1.4±1.7	0.0-4.0	-	NS	0.8
	3	[redacted] BIT (600)	M	0.6±0.9	0.0-2.0	NS	-	0.8
			F	0.0±0.0	0.0-0.0		-	0.7
			Group	0.3±0.7	0.0-2.0	-	NS	0.7
	4	[redacted] BIT	M	2.1±1.1	0.9-3.9	*	NS	0.8
			F	0.3±0.5	0.0-1.0		NS	0.8

Sacrifice Time	Group	Treatment (mg/kg)		MnP cells/ 1000		S.S.		P:M
				Mean ± SD	Range	MvsF	Treated vs Control	
		(1200)	Group	1.3±1.3	0.0-3.9	-	-	0.8
	5	CBC (30)	M	38.4±5.3	30.7-44.8	NS	-	0.5
			F	58.1±23.1	28.4-87.0		-	0.7
			Group	48.2±18.9	28.4-87.0	-	**	0.6
48 Hours		Vehicle (-)	M	1.8±2.5	0.0-5.9	NS	-	0.7
			F	0.4±0.9	0.0-2.0		-	0.8
			Group	1.1±1.9	0.0-5.9	-	NS	0.7
		[redacted] BIT (1200)	M	1.5±1.3	0.0-3.0	NS	-	0.7
			F	1.5±1.9	0.0-4.0		-	0.7
			Group	1.5±1.5	0.0-4.0	-	NS	0.7
72 Hours	1	Vehicle (-)	M	0.4±0.8	0.0-1.8	NS	-	0.7
			F	1.5±2.1	0.0-3.9		-	0.7
			Group	1.0±1.6	0.0-3.9	-	-	0.7
	4	[redacted] BIT (1200)	M	0.6±0.9	0.0-1.9	NS	-	0.7
			F	0.3±0.5	0.0-1.0		-	0.7
			Group	0.4±0.7	0.0-1.9	-	NS	0.7

MnP: Micronucleated polychromatic erythrocytes
P:M:Ratio of polychromatic to mature erythrocytes
CBC: Chiorambucil
Vehicle: Corn oil
S.S: Statistical significance of the frequency of micronucleated polychromatic erythrocytes (Males vs Females, and Treated vs Vehicle Controls)
NS Not significant, $p > 0.05$
* Significant, $0.05 > p > 0.01$
* * Highly significant, $p < 0.01$

Section A6 Toxicological and Metabolic Studies

Subsection A6.6.5 Genotoxicity *in vivo*

Annex Point IIA VI.6.6.5 ***In vivo UDS Assay (Rat Hepatocytes)***

			Official use only
		1 REFERENCE	
1.1	**Reference**	[redacted] 2001; [redacted] (BIT): Measurement of Unscheduled DNA Synthesis in Rat Liver using an *In vivo/In vitro* Procedure. [redacted]., Report No. 1803/21-D6173	
1.2	**Data protection**	Yes	
1.2.4	Data owner	Arch Chemicals Inc and Clariant Production UK Ltd	
1.2.5	Company with letter of access	Thor GmbH	
1.2.6	Criteria for data protection	Data on existing substance for first entry in to Annex I.	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	OECD 486 (1997), UKEMS (1993)	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	[redacted]	
3.1.2	Specification	Deviating from specification given in section 2 as follows: The Test Substance employed was pre-dried technical grade active substance.	
3.1.2.1	Purity	93.1%	
3.1.2.2	Stability	The expiry date of the test item was stated as 19 October 2002 (the experimental work was completed on 19 January 2001)	
3.1.2.3	Maximum tolerable dose	1400 mg [redacted] kg body weight	
3.2	**Test Animals**		
3.2.1	Species	Rat	
3.2.2	Strain	Han Wistar	

Section A6 Toxicological and Metabolic Studies

Subsection A6.6.5 Genotoxicity *in vivo*

Annex Point IIA VI.6.6.5 ***In vivo UDS Assay (Rat Hepatocytes)***

3.2.3 Source [redacted]

3.2.4 Sex Male

3.2.5 Age/weight at study initiation Weight at start of Range Finder Study: 188-258 g

Weight at start of Main Study: 186-224 g

3.2.6 Number of animals per group

Preliminary Range Finding Test

Groups of three animals were dosed once with the following concentrations:

700, 1000, 1400 and 2000 mg/kg [redacted]

Main Test

Treatment	Dose (mg/kg)	Dose Volume (ml/kg)	Number of Animals Dosed*	
			Experiment 1 (12-14 hour)	Experiment 2 (2-4 hour)
0.5% MC	0	10	4	4
[redacted] (BIT)	560	10	4	3**
[redacted] (BIT)	1400	10	4	5**
2-AAF	75	10	4	-
DMN	10	10	-	4

* Cultures were made from three animals in each dose group or from all surviving animals (there were two mortalities at 1400 mg/kg in the 12-14 hour experiment and therefore slides from only two animals were examined).

** In view of potential for mortalities at the top dose (following from mortalities in the 12-14 hour study), one animal was reassigned from the low dose group to the high dose group.

MC: Methyl cellulose

2-AAF: Acetamidofluorene

DMN:Dimethylnitrosamine

Section A6 Toxicological and Metabolic Studies

Subsection A6.6.5 Genotoxicity *in vivo*

Annex Point IIA VI.6.6.5 ***In vivo UDS Assay (Rat Hepatocytes)***

3.2.7	Control animals	Negative Control: 0.5% methyl cellulose Positive Control: Acetamidofluorene (2-AAF) and Dimethylnitrosamine (DMN)
3.3	**Administration/ Exposure**	Oral
3.3.1	Number of applications	One application
3.3.2	Interval between applications	Not applicable
3.3.3	Postexposure period	Range Finder Study 48 hour Main Study Experiment 1: 12-14 hours Experiment 2: 2-4 hours
3.3.4	Type	Gavage
3.3.5	Concentration	Range Finder Study 700, 1000, 1400, and 2000 mg/kg body weight Main Study (Experiment 1 and 2) 560 and 1400 mg/kg body weight
3.3.6	Vehicle	Methyl cellulose
3.3.7	Concentration in vehicle	

Experiment	Dose Volume (mL/kg)	Concentration of Dosing Preparation (mg/mL)	Dose Administered (mg/kg body weight)
Range-Finder	10	70.0	700
		100.0	1000
		140.0	1400
		200.0	2000
Main Study	10	56.0	560
		140.0	1400

3.3.8	Total volume applied	10 mL/kg body weight. Animals were weighed before dosing and the volume of vehicle, test article preparation or positive control solution to be administered was calculated based on a dose volume of 10 mL/kg. Samples were saved from the range-finder and main study dosing preparations and were analysed for homogeneity and achieved concentration, respectively. This analysis was performed at [redacted]
3.3.9	Controls	Negative Control • 0.5% methyl cellulose Positive Controls • Acetamidofluorene (2-AAF) suspended in corn oil at a concentration of 7.5 mg/L was used in Experiment 1 in the Main Test. • Dimethylnitrosamine (DMN) dissolved in purified water at a concentration of 1.0 mg/L was used in Experiment 2 in the Main Test. Both positive controls were administered at a dose volume of 10 mL/kg body weight.
3.4	**Examinations**	
3.4.1	Clinical signs	Preliminary Test: Clinical observations were made during the two day post-exposure period to assess the toxicity of [redacted] Main Test: Clinical observations were made during the post exposure period in both experiments in the main test.
3.4.2	Tissue	Approximately 12-14 hours (Experiment 1) or 2-4 hours (Experiment 2) after dosing, animals were anaesthetised with Halothane and their livers perfused with collagenase to provide a primary culture of hepatocytes (approximately 1.5 x 10^5 viable cells/mL). Cultures were made from three animals in each dose group (or in the case of the 1400 mg/kg dose group in the 12-14 hour experiment, from the two surviving animals) and were treated with [^3H]-thymidine. Six slides from each animal were prepared with fixed hepatocytes and of these, three were dipped in photographic emulsion to prepare autoradiograms.

Section A6 Toxicological and Metabolic Studies

Subsection A6.6.5 Genotoxicity *in vivo*

Annex Point IIA VI.6.6.5 ***In vivo UDS Assay (Rat Hepatocytes)***

Number of animals:

12-14 Hour Experiment (Experiment 1)

Hepatocytes were prepared from three of the four animals in the 0.5% Methyl cellulose (negative control), acetamidofluorene (positive control) and 560 mg/kg [redacted] dose groups. Due to mortalities (two out of four animals) at the 1400 mg/kg [redacted] dose group in this experiment, it was only possible to prepare hepatocytes from two animals.

2-4 Hour Experiment (Experiment 2)

Hepatocytes were prepared from three of the four animals in the 0.5% Methyl cellulose (negative control) and dimethylnitrosamine (positive control) dose groups.

In view of the mortalities at the top dose in the 12-14 hour experiment, one animal was reassigned from the 560 mg/kg dose group to the 1400 mg/kg dose group. Hepatocytes were therefore prepared from all three animals in the 560 mg/kg dose group and three of five animals in the 1400 mg/kg dose group (there were no mortalities observed) in the 2-4 hour experiment.

Number of cells:

100 cells were analysed per animal, where possible using two out of three slides in each case.

Time points:

Main Study, Experiment 1: 12-14 hours after dosing.

Main Study, Experiment 2: 2-4 hours after dosing.

Type of cells

Hepatocytes with normal morphology (see criteria listed below Net grain counting)

Parameters:

Net Grain Count (NNG)

Slides were examined microscopically after development of the emulsion and staining, and the net grain count (NNG), the number of grains present in the nucleus minus the mean number of grains in three equivalent areas of cytoplasm, was determined. Each slide was examined to ensure that the culture was viable prior to cell analysis.

100 cells were analysed per animal, where possible using two out of three slides in each case. The following criteria were used for cell analysis:

- Only cells with normal morphology were scored.
- Isolated nuclei with no surrounding cytoplasm were not scored.
- Cells without nuclear and/or cytoplasmic graining were not scored.
- Cells with unusual staining artefacts were not scored.

- Heavily labelled cells in S-phase were not scored.
- All other normal cells, 100 per animal were scored.
- All slides were analysed blind (coded).

Treatment of data

The following were calculated for each slide, animal and dose point:

- The population average NNG and standard deviation (SD)
- The percentage of cells responding or in repair (with ≥ 5 NNG)
- The population average cytoplasmic and nuclear grain count.

Acceptance Criteria

The study was considered valid if the negative control animals had a group mean NNG value that did not exceed the upper limit of the historical range. The positive control treatments should have group mean values of five or more NNG with 50% or more cells having NNG counts of five or greater.

Evaluation Criteria

The test article would be considered as positive in this assay if, at any dose and at either time point:

- The test article yields a group mean NNG value greater than 0 NNG and 20% or more of the cells are in repair (mean NNG values ≥ 5)
- An increase above solvent control levels is seen in both NNG and the percentage of cells in repair.

Cytoplasmic and nuclear grain count values as well as the concurrent negative control data are considered in relation to the overall NNG values of cultures from treated animals.

If the test article fails to induce UDS at any dose tested after both 2-4 and 12-14 hours exposure, it would be considered clearly negative in this system.

Section A6 Toxicological and Metabolic Studies

Subsection A6.6.5 Genotoxicity *in vivo*

Annex Point IIA VI.6.6.5 ***In vivo UDS Assay (Rat Hepatocytes)***

3.5 Further remarks

Analysis for Homogeneity and Achieved Concentration of Dosing Solutions

Homogeneity was determined at concentrations 56 and 140 mg/mL. Three samples were removed from the top and three from the bottom of each concentration after preparation.

Duplicate samples from two different batches of dosing solution (prepared for Experiment 1 and 2) at 56 and 140 mg/mL were analysed for confirmation of concentration of [redacted]

The samples were analysed with calibration standard solutions (prepared using [redacted] by HPLC with UV detection using a validated method.

4 RESULTS AND DISCUSSION

4.1 Clinical signs

Preliminary Study:

During a 2 day post-dose observation period the following clinical signs were observed:

700 mg/kg: Piloerection and lethargy. Some weight loss.

1000 mg/kg: Piloerection, abnormal breathing and lethargy. Some weight loss.

1400 mg/kg: Piloerection and lethargy. Some weight loss.

2000 mg/kg: Piloerection, eye closure, abnormal breathing and lethargy. One animal was killed *in extremis* shortly after dosing and one animal was found dead one day after dosing. In the remaining animal some weight loss was observed.

As mortalities were seen at 2000 mg/kg, a dose of 1400 mg/kg was considered representative of a maximum tolerated dose (and therefore the maximum dose for the main experiments). A lower dose of 560 mg/kg was also tested.

Main Study

Clinical signs of piloerection and lethargy were observed at the 1400 mg/kg dose level. Furthermore, the day after dosing at 1400 mg/kg in the 12-14 hour experiment, two animals were found dead. No clinical signs were observed in the 560 mg/kg dose group.

4.2 Haematology / Tissue examination

Hepatocytes

Section A6 Toxicological and Metabolic Studies

Subsection A6.6.5 Genotoxicity *in vivo*

Annex Point IIA VI.6.6.5 ***In vivo UDS Assay (Rat Hepatocytes)***

4.3	**Genotoxicity**	No Treatment with [redacted] at doses up to 1400 mg/kg yielded NNG values less than zero, producing group mean NNG values over the two experiments in the range of -2.5 to -0.1, values below the threshold of zero NNG required for a positive response. No cells were seen in repair at any dose of [redacted] Negative (vehicle) control animals gave group mean NNG values of -2.3 and 0 in Experiments 1 and 2, respectively. These values did not exceed the upper limit of the historical negative control range and only 0 to 0.3% cells in experiments 1 and 2 were in repair. Group mean NNG values were increased by 2-AAP and DMN treatment to ≥ 5 and more than 50% cells found to be in repair. In this study the vehicle control NNG value was consistent with both published and historical control data, and the system was shown to be sensitive to two known DNA damaging agents requiring metabolism for their action. The assay was therefore accepted as valid.
4.4	**Other**	Homogeneity At 56 mg/kg the accuracy of the duplicate samples analysed ranged from 104 to 111% with a coefficient of variation of 2.1%. At 140 mg/kg the accuracy of the duplicate samples ranged from 101 to 107% with a coefficient of variation of 2.2%. Confirmation of Concentration of [redacted] in Dosing Solutions Analysis of duplicate samples from dosing solutions prepared on two separate occasions (for Experiment 1 and 2) gave results of 78, 77, 61 and 62% of the nominal concentration at 56 mg/kg and 66, 68, 73 and 73% of the nominal concentration at 140 mg/kg. There was no obvious explanation for these low results given by the analytical facility. The homogeneity of formulations had been good and all formulations were individually prepared thus ruling out potential dilution errors. Accordingly, it was not considered likely by the author that the low results reflected errors in test article preparation. Furthermore, the test article was tested up to the limits of toxicity, hence the low formulation results were not considered to have prejudiced the validity of the study.
	5 APPLICANT'S SUMMARY AND CONCLUSION	
5.1	**Materials and methods**	In an initial toxicity range-finder study, groups of three male rats were dosed once with 700, 1000. 1400 and 2000 mg/kg [redacted] (BIT). Mortalities were seen at 2000 mg/kg and therefore a dose of 1400 mg/kg was considered representative of a maximum tolerated dose. Groups of at least three male rats were treated once with the solvent 0.5% methyl cellulose; [redacted] (BIT) at 560 mg/kg or

1400 mg/kg; or the required positive control, by oral gavage, at a dose volume of 10 mL/kg. The positive controls used were 75 mg/kg 2-acetamidofluorene (2-AAF) suspended in corn oil (12-14 hour experiment) and 10 mg/kg dimethylnitrosamine (DMN) dissolved in purified water (2-4 hour experiment).

Approximately 12-14 hours (Experiment 1) or 2-4 hours (Experiment 2) after dosing, animals were killed and their livers perfused with collagenase to provide a primary culture of hepatocytes. Cultures were made from three animals in each dose group (or in the case of the 1400 mg/kg dose group in the 12-14 hour experiment, from the two surviving animals) and were treated with [^{3}H]-thymidine. Six slides from each animal were prepared with fixed hepatocytes and of these three were dipped in photographic emulsion to prepare autoradiograms. Slides were examined microscopically after development of the emulsion and staining, and the net grain count (NNG), the number of grains present in the nucleus minus the mean number of grains in three equivalent areas of cytoplasm, was determined for one hundred cells with normal morphology (from two of the three slides prepared). For each slide, animal and dose point, the population average NNG and percentage of cells responding or in repair (NNG of ≥ 5) was calculated.

The data were evaluated for indication of [redacted] induced UDS. The criteria for a positive result were if the [redacted] (at any dose at either post dose time point) yielded group mean NNG values of zero or above and ≥ 20% of cells had mean NNG values of ≥ 5. The data would also be indicative of a positive result if an increase above the solvent control levels was seen in both NNG and the percentage in repair.

Section A6 Toxicological and Metabolic Studies

Subsection A6.6.5 Genotoxicity *in vivo*

Annex Point IIA VI.6.6.5 ***In vivo UDS Assay (Rat Hepatocytes)***

5.2 Results and discussion

In the preliminary study during the 2 day post-dose observation period piloerection, weight loss, lethargy and abnormal breathing (1000 mg/kg dose level only) were observed at 700, 1000 and 1400 mg/kg dose levels. At 2000 mg/kg, piloerection, eye closure, abnormal breathing and lethargy were observed. One animal was killed *in extremis* shortly after dosing at 2000 mg/kg and one animal from this dose group was found dead one day after dosing. In the remaining animal some weight loss was observed.

As mortalities were seen at 2000 mg/kg, a dose of 1400 mg/kg was considered representative of a maximum tolerated dose. The maximum dose level of 1400 mg/kg was therefore selected for the main study which was tested together with a dose rate of 560 mg/kg.

In the main study, clinical signs of piloerection and lethargy were observed at the top dose tested, 1400 mg/kg, in both experiments. On the day after dosing with 1400 mg/kg [redacted] in the 12-14 hour experiment, two animals (out of four animals) were found dead. No clinical signs were observed in the 560 mg/kg dose group.

Negative (vehicle) control animals gave group mean NNG values of -2.3 and 0 in experiments 1 and 2, respectively. These values did not exceed the upper limit of the historical negative control range and only 0 to 0.3% cells in experiments 1 and 2 were in repair. Group mean NNG values were increased by 2-AAP and DMN treatment to ≥ 5 and more than 50% cells found to be in repair. The vehicle control NNG value was consistent with both published and historical control data, and the system was shown to be sensitive to two known DNA damaging agents requiring metabolism for their action. The assay was therefore accepted as valid.

Treatment with [redacted] at doses up to 1400 mg/kg yielded NNG values less than zero, producing group mean NNG values over the two experiments in the range of -2.5 to -0.1, values below the threshold of zero NNG required for a positive response. No cells were seen in repair at any dose of [redacted] (BIT).

It may be noted that due to mortalities seen at the top dose tested in the 12-14 hour experiment, it was only possible to analyse UDS from two animals. In view of the clear negative results obtained in this assay, this was not considered to have prejudiced the validity of the study.

The data obtained in this study indicate that oral treatment of male rats dosed once with 540 or 1400 mg/kg [redacted] did not result in increased UDS in hepatocytes isolated approximately 12-14 or 2-4 hours after dosing.

5.3 Conclusion

When treated orally once with [redacted] at doses up to 1400 mg/kg, male rats showed no induction of UDS in hepatocytes isolated *ex vivo* approximately 12-14 or 2-4 hours after dosing. It is concluded that [redacted] had no genotoxic activity detectable in this test system under the experimental conditions

		employed. The study can be considered to be compatible with OECD 486. Although UDS analysis was not performed on three animals in the top dose group (due to a mortality) in the 12-14 hour test, it is not considered to affect the validity of the study since the results from the UDS analysis performed were clearly negative.
5.3.1	Reliability	1
5.3.2	Deficiencies	No

Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*September 2008*
Materials and Methods	*Applicant's version is accepted.*
Results and discussion	*Applicant's version is accepted*
Conclusion	*Applicant's version is adopted.*
Reliability	*1*
Acceptability	*Acceptable*
Remarks	

Table 6_6_5-1: Summary of Group Mean Net Grain Values (12-14 hour Experiment)

Dose (mg/kg)	Compound	Net Grain Count (NNG)		Percentage of Cells In Repair (NNG ≥ 5)		Net Grain Count of Cells in Repair	
		Mean	SD	Mean	SD	Mean	SD
0	0.5% MC	-2.3	0.4	0.3	0.6	5.3	-
560	PPP	-2.5	0.4	0	-	-	-
1400	PPP	-2.4	1.4	0	-	-	-
75	2-AAF	11.4*	1.9	84.0*	12.0	12.6	1.0

MC: Methyl cellulose
PPP: [redacted]
2-AAF: Acetamidofluorene
* Value within observed range of historical control

Table 6_6_5-2: Summary of Group Mean Net Grain Values (2-4 hour Experiment)

Dose (mg/kg)	Compound	Net Grain Count (NNG)		Percentage of Cells In Repair (NNG ≥ 5)		Net Grain Count of Cells in Repair	
		Mean	SD	Mean	SD	Mean	SD
0	0.5% MC	0.2	0.1	0	-	-	-
560	PPP	-0.1	0.1	0	-	-	-
1400	PPP	-0.2	0.2	0	-	-	-
10	DMN	5.4	0.0	56.1*	3.4	8.0	0.2

MC: Methyl cellulose
PPP: [redacted]
DMN:Dimethylnitrosamine
* Value within observed range of historical control

Section A6 **Subsection A6.6.6** **Annex Point IIA6.6.6**	**Toxicological and Metabolic Studies** **IF POSITIVE IN 6.6.4 THEN A TEST TO ASSESS POSSIBLE GERM CELL EFFECTS MAY BE REQUIRED**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ] **Limited exposure [ ]**	**Technically not feasible [ ]** **Scientifically unjustified [X]** **Other justification [ ]**	
Detailed justification:	This study is only required if Genotoxicity *In vivo* returns a positive result. In the test reported in Section 6.6.4, There was no evidence of induced chromosomal or other damage leading to micronucleus formation in polychromatic erythrocytes of treated mice 24, 48 or 72 hours after oral administration of BIT. Therefore, non-inclusion of this data is justified on the basis that the criteria for requirement are not met.	
Undertaking of intended data submission [ ]		

Evaluation by Competent Authorities

EVALUATION BY RAPPORTEUR MEMBER STATE

Date	*September 2008*
Evaluation of applicant's justification	*Applicant's justification is accepted.*
Conclusion	*Applicant's is exempted to the test to assess possible germ cell effects may be required.*
Remarks	

Section A6	**Toxicological and Metabolic Studies**	
Subsection A6.6.7 **Annex Point IIA6.6.7**	**IF THE RESULTS ARE NEGATIVE FOR THE THREE TESTS 6.6.1, 6.6.2 AND 6.6.3, THEN FURTHER TESTING IS NORMALLY ONLY REQUIRED IF METABOLITES OF CONCERN ARE FORMED IN MAMMALS**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ] **Limited exposure [ ]**	**Technically not feasible [ ] Scientifically unjustified [X]** **Other justification [ ]**	
Detailed justification:	This study is only required if metabolites of concern are presented that require further investigation for genotoxicity. From the data presented in Section 6.2 and 6.6, it is clear that there are no metabolites of concern that would require further investigation for genotoxicity. Therefore, non-inclusion of this data is justified on the basis that the criteria for requirement are not met.	
Undertaking of intended data submission [ ]		
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*September 2008*	
Evaluation of applicant's justification	*Applicant's justification is accepted.*	
Conclusion	*Applicant is exempted to assay BIT metabolites.*	
Remarks		

Section 6	**Toxicological and Metabolic Studies**	
Subsection A6.7	**Carcinogenicity study**	
Annex Point IIA6.7		
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ]	**Technically not feasible [ ]** **Scientifically unjustified [X]**	
Limited exposure [ ]	**Other justification [ ]**	
Detailed justification:	This justification for non-submission of data is a brief outline of the detailed discussion presented in the following Expert Report; [redacted] 2007; Review of toxicological Data on 1,2 Benzisothiazolin 3 one (BIT) and other isothiazolinones; Report: SJB/BIT/150507. The full report is presented in the BIT Dossier IVA6.5. **Subchronic Toxicity of Isothiazolinones** Isothiazolinone derivatives are consistent qualitatively in their toxicological profile. The primary effects from exposure to multiple species are a slight reduction in body weight gain, inflammation at the initial site of contact regardless of the route of exposure, and slight increase in organ weight (liver and kidney). The increase in liver and kidney weight is of questionable toxicological significance since there was no associated histopathological change in these organs. Emesis was observed in the species that has this ability, but it is likely associated with a local irritant effect. **Summary of Genotoxicity of BIT** In the Ames assay, isolated increases in revertants were observed in some Salmonella strains, although the lack of reproducibility and dose response usually seen suggests that this is not a true chemically mediated effect. This is supported by the negative response seen in the L5178Y mammalian cell gene mutation assay. Negative responses were also seen in vitro in a cell transformation assay and a UDS assay. BIT produced a negative response in the in vivo mouse bone marrow micronucleus assay and the rat-liver UDS assay, indicating that the compound is not metabolized to a mutagenic species in the whole animal. BIT did produce chromosomal aberrations in two in vitro cell systems, but the lack of chromosomal aberrations observed in vivo indicates that chromosomal damage would not occur in the whole animal. The conclusion, based on the results from this battery of assays, is that BIT presents no potential to produce genetic damage to mammalian cells in vivo. The lack of potential of BIT to induce genetic damage is similar to other isothiazolinones. These isothiazolinones have been evaluated for genotoxic potential in a number of short term assays including end points of gene mutation, chromosomal damage and DNA repair. **Summary of Metabolism and Disposition of Isothiazolinones** Isothiazolinones, including BIT, are absorbed rapidly from oral administration and excreted rapidly as well, primarily in the urine. These	

Section 6 **Subsection A6.7** **Annex Point IIA6.7**	**Toxicological and Metabolic Studies** **Carcinogenicity study**	
	chemicals are not distributed preferentially to any organ and there is no tendency for bioaccumulation. For those isothiazolinones containing the aromatic ring, the available data indicate that the metabolism follows the path of ring opening with oxidation of the sulphur and methylation of the nitrogen if not already occupied by an alkyl group. For those compounds without the aromatic ring, the isothiazolinone ring undergoes more extensive catabolism. The metabolism of isothiazolinones is rapid and virtually complete with little to no excretion of the parent compound. **Structure-Activity Relationship analysis for BIT** BIT was assessed for carcinogenic potential through structure-activity relationship (SAR) analyses. Based on the results from the 4 models, BIT is predicted to lack the potential to cause cancer. Thus, it has a high probability of not inducing cancer in either rats or mice. The estimate from each model is derived from a structural comparison of BIT to chemicals previously assessed for carcinogenicity. The estimates are robust since none is based on the results of a single model but rather on analyses using Bayes' Theorem to combine the Rat/MIT CMIT and the Mouse/OIT predictions. Moreover, individual predictions are not based on the occurrence of a single descriptor (i.e., fragment) but rather multiple descriptors. And finally, each fragment is derived from several compounds with similar carcinogenic or non-carcinogenic activity. **Summary** The toxicological profile of BIT has been compared to that of other isothiazolinones to demonstrate the similarity in the toxicology for members of this chemical class. Illustration of toxicological similarity between isothiazolinones allows the reasoned judgment that carcinogenicity and chronic toxicity data should not be required for BIT.	
Undertaking of intended data submission [ ]		
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*October 2008*	
Evaluation of applicant's justification	*Applicant version is accepted.*	
Conclusion	*Applicant's is exempted of the carcinogenity studies.*	

Section 6	**Toxicological and Metabolic Studies**
Subsection A6.7	**Carcinogenicity study**
Annex Point IIA6.7	
Remarks	

Section A6 Toxicological and Metabolic Studies

Subsection A6.8.1/1 TERATOGENICITY STUDY

Annex Point IIA VI.6.8.1 ***Rat Oral Gavage***

			Official use only
		1 REFERENCE	
1.1	**Reference**	[redacted] 1988; [redacted] Teratogenicity Study in the Rat. [redacted] Report No. [redacted]P/2297.	
1.2	**Data protection**	Yes	
3.1.1	Data owner	Arch Chemicals Inc	
3.1.2	Companies with letter of access	Clariant Production UK Ltd and Thor GmbH	
3.1.3	Criteria for data protection	Data on existing substance for first entry into Annex I.	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	Methods used comparable to EC B.31	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	[redacted]	
3.1.2	Specification	As given in section 2	
3.1.2.1	Purity	73.4 % Correction was made for this purity when preparing the dosing formulations.	
3.1.2.2	Stability	The chemical stability of BIT in the vehicle was determined by re-analysis of the dosing formulation of the lowest and highest dose concentrations (nominally 1.0 and 10.0 mg/mL) after intervals of 14, 20 and 34 days.	
3.2	**Test Animals**	Non-entry field	
3.2.1	Species	Rat	
3.2.2	Strain	Alpk:APfSD (Wistar-derived)	

Section A6 Toxicological and Metabolic Studies

Subsection A6.8.1/1 TERATOGENICITY STUDY

Annex Point IIA VI.6.8.1 ***Rat Oral Gavage***

3.2.3	Source	[redacted]
3.2.4	Sex	Female
3.2.5	Age/weight at study initiation	The average weights from Day 1are tabulated as follows:

Weight (g)	Dose Level of [redacted] (mg/kg/day)			
	0 (Control)	10	40	100
Group Average (Day 1)	251.5	252.1	255.8	254.9

On arrival (Day 1 of gestation) the animals were approximately 12 weeks old.

3.2.6	Number of animals per group	24
3.2.7	Control animals	Yes
3.2.8	Mating period	Virgin female rats were paired overnight at the Breeding Unit with unrelated males of the same strain. On the following morning, vaginal smears from these females were examined for the presence of sperm. The day when spermatozoa were detected was designated Day 1 of gestation and on this same day successfully mated females were delivered to the experimental unit at the [redacted]
3.3	**Administration/ Exposure**	Oral
3.3.1	Duration of exposure	10 days The rats were dosed on Days 7-16 (inclusive) of the gestation period.
3.3.2	Postexposure period	6 Days The post exposure period was Days 17-22 (the animals were sacrificed on Day 22).

Section A6 Toxicological and Metabolic Studies

Subsection A6.8.1/1 TERATOGENICITY STUDY

Annex Point IIA VI.6.8.1 ***Rat Oral Gavage***

3.3.3	Type	Oral gavage All animals were dosed with l mL of dosing suspension per l00 g bodyweight using a 5 mL disposable syringe and a stainless steel 16 gauge cannula.
3.3.4	Concentration	The dose levels are detailed in the table below.

Group	Dose Level of [redacted] as BIT (mg/kg bw/day)	Animal Numbers
1	0	1-24
2	10	25-48
3	40	49-72
4	100	73-96

A sample of each preparation was analysed prior to the start of dosing to verify the achieved concentrations of the active ingredient of [redacted] (BIT) in the vehicle.

Aliquots dispensed at the start, approximately in the middle and at the end of the sub-division process of the bulk formulations of the 1.0 and 10.0 mg/mL dose levels were analysed to determine homogeneity.

3.3.5	Vehicle	0.5% (w/v) HMPC in 0.1% (w/v) aqueous Polysorbate 80.
3.3.6	Concentration in vehicle	[redacted] was formulated in vehicle and the concentration adjusted to give a constant volume of l mL/l00 g bodyweight for each dose level.
3.3.7	Total volume applied	[redacted] was formulated in vehicle and the concentration adjusted to give a constant volume of l mL/l00 g bodyweight for each dose level.
3.3.8	Controls	Yes
3.4	**Examinations**	
3.4.1	Body weight	The bodyweight of each animal was recorded on Days 1 and 4; daily from Days 7-16 (inclusive); and on Days 19 and 22 of gestation.

Section A6 Toxicological and Metabolic Studies

Subsection A6.8.1/1 TERATOGENICITY STUDY

Annex Point IIA VI.6.8.1 ***Rat Oral Gavage***

3.4.2	Food consumption	CTI diet (supplied by Special Diets Services Limited, Stepfield, Witham, Essex, UK) and tap water (*via* an automatic watering system) were available *ad libitum* except when food consumption was being measured. The amount of food consumed by each animal over three day periods was measured by giving a weighed quantity of food on Days 1, 4, 7, 10, 13, 16 and 19 and calculating the amount consumed from the residue on Days 4, 7, 10, 13, 16, 19 and 22, respectively.
3.4.3	Clinical signs	All animals were checked on arrival to ensure that they were physically normal externally. The animals were subsequently observed daily and any substantial changes in behaviour or clinical condition recorded. A more detailed examination including the observation of no abnormalities detected was made during the dosing period and on other days when the animals were weighed.
3.4.4	Examination of uterine content	On Day 22 of gestation all the animals were killed by over-exposure to halothane BP vapour (Fluothane, ICI Pharmaceuticals, Macclesfield, Cheshire, UK).
3.4.5	Maternal organ weights	A post mortem examination, at which tissues were examined macroscopically, was performed on all animals. Liver, ovaries, kidney, stomach, small intestines, caecum, colon and rectum were also examined for abnormalities (the organs examined were not detailed in the report however the organs stated were reported as macroscopic findings and therefore these organs have been listed).
3.4.5.1		The intact gravid uterus (minus ovaries and trimmed free of connective tissue) was removed and weighed. The ovaries and uterus were then examined and the following data recorded: • Number of *corpora lutea* in each ovary. • Number and position of implantations subdivided into live foetuses, early intra-uterine deaths and late intra-uterine deaths Intra-uterine deaths were classified as follows: • Early intra-uterine deaths showed decidual or placental tissue only. • Late intra-uterine deaths showed embryonic or foetal tissue in addition to placental tissue. The implantations were assigned letters of the alphabet to identify their position *in utero* starting at the ovarian end of the left horn and

ending at the ovarian end of the right horn.

3.4.5.2 Skeleton

3.4.5.3 Soft tissue

The litter sizes and number of dead foetuses were recorded and each foetus was weighed and individually identified.

Each foetus was examined for external abnormalities and for cleft palate.

3.5 Further remarks

The stained foetal skeletons were examined for abnormalities and the degree of ossification was assessed. The individual bones of the *manus* and *pes* were assessed as detailed below.

Scale for Assessment of Skeletal Ossification of the *Manus* and *Pes*

1 (good): Metacarpals/metatarsals and first and third row of phalanges fully ossified (or one phalanx partially ossified).

2: Metacarpals/metatarsals fully ossified. First or third row of phalanges ossified, although an occasional phalanx (approximately up to four) may be partially ossified.

3: Metacarpals/metatarsals fully or occasionally partially ossified. First row phalanges either partially or not ossified together with third row of phalanges either partially or fully ossified.

4: (poor) Metacarpals/metatarsals - some either partially or not ossified plus first row of phalanges usually not ossified and third row of phalanges partially ossified.

4 RESULTS AND DISCUSSION

4.1 Maternal toxic effects

Clinical Observations

One animal (number 91) from the 100 mg/kg/day group was found dead on Day 17. There were no other mortalities.

There was a dose-related increase (both in incidence of observations and numbers of animals affected) in abnormal respiratory noise in the 40 and 100 mg/kg/day groups.

Otherwise the findings were of a type commonly found in Alpk:APfSD rats and the incidence was not affected by treatment with [redacted]

Bodyweight Gain

Mean bodyweight gain showed a dose-related reduction during the dosing period (Days 7-16) in animals receiving 40 mg/kg/day (not statistically significant when compared to the control group) and 100 mg/kg/day (there was a statistically significant difference from the control at the 1% level).

Some individual animals in the 100 mg/kg/day showed weight loss (up to 23g) during Days 10-13 (mean body weight for 100 mg/kg/day

dose group showed a statistically significant difference from the control at the 1% level) and Days 13-16 (mean body weight for 100 mg/kg/day dose group was not statistically significantly different from the control group). There were also individual animal weight losses observed in the 40 mg/kg/day group during Days 10-13 and 13-16; however there was no statistically significant difference in the mean bodyweights for this group and the control group.

The mean weight gain at 100 mg/kg/day during the post dosing period was similar to controls (mean body weight for 100 mg/kg/day dose group was not statistically significantly different from the control group) although the effects seen in some individual animals during dosing persisted during Days 16-19.

Food Consumption

Food consumption was reduced in the 100 mg/kg/day group during Days 10-16 of the dosing period and Days 16-19 of the post dosing period (there were statistically significant differences in the mean body weights from Day 10-13, Day 13-16 and Day 16-19 when compared to the control group, at the 1% level). A few animals were more severely affected than others during this period.

A few individual animals in the 40 mg/kg/day group showed reduced food consumption during Days 13-16 of the dosing period and during the post dosing period. This is reflected in the marginal (although not statistically significant) reduction in mean values for this period.

The low individual food consumption values at 40 or 100 mg/kg/day during Days 10-16 correlated with the animals showing either weight loss or very low weight gain.

Macroscopic Findings at Post Mortem

Four animals in the 100 mg/kg/day group showed stomach lesions at Day 22. There were no other findings at termination which are considered to be related to treatment.

The animal dosed at 100 mg/kg/day found dead on Day 17 showed evidence of mis-dosing into the lung. This animal also had stomach lesions.

4.2 Teratogenic / embryo toxic effects

Litter Data

The only evidence for a treatment-related effect on litter data parameters was 3.6% reduction in mean foetal weight for the 100 mg/kg/day group (this reduction was of statistical significance when compared to the control group, at the 5% level). This was exaggerated by one litter (from female 96) with a mean foetal weight of 3.7 g. [Female 96 gained only 52 g during gestation (compared with the group mean of 128 g) despite having a litter of 13 foetuses and may have shown a high individual susceptibility to the toxicity of [redacted]

Section A6 Toxicological and Metabolic Studies

Subsection A6.8.1/1 TERATOGENICITY STUDY

Annex Point IIA VI.6.8.1 ***Rat Oral Gavage***

Foetal Assessments

Classification of Foetal Observations

For the first stage evaluation of data, foetal observations are classified as major defects, minor defects or variants. These classifications are not used as a definitive evaluation and are dependent on species and strain and may be re-classified on the basis of the results obtained on that study. Normally, the category assigned to any observation is based on the following guidelines.

Major defect - Rare or marked malformations that would normally be incompatible with successful survival.

Minor defect - Small changes that would not normally impair survival and that occur at a moderate to low frequency in the strain.

Variant - Common observations that are not normally deleterious.

Major Defects

Major abnormalities were seen in 10 foetuses with 0, 3, 4 and 3 in the control, 10, 40 and 100 mg/kg/day groups, respectively (2 abnormalities in one foetus in the 100 mg/kg/day group). These defects included abdominal ascites, cysts attached to the liver, *situs inversus totalis* of the torso, moderately dilated lateral ventricles of the brain, major vertebral defects, multiple minor skeletal defects of the limbs and ribs, cleft lip, anophthalmia and fused mandibles. There was no evidence for a relationship to treatment in the incidence of any specific major defect.

Minor Defects

The overall incidence of minor external/visceral defects was lower in all treated groups due to a high control incidence of blood clots attached to the intestines, the significance of which is uncertain. Excluding this anomaly (when it was the only anomaly in a foetus), there was no effect on the incidence of foetuses with minor external/visceral defects (25, 20, 26 and 17 in the control, 10, 40 and 100 mg/kg/day groups respectively).

There was some intergroup variation in the overall incidence of minor skeletal defects both above and below the control value with no evidence for an effect of compound.

Individual defects to show a statistically significant difference from control were as follows:-

The incidence of non-ossified cervical centra (2nd-5th vertebrae) showed a dose-related increase at 100 mg/kg/day, with significant differences when compared to the control group (at 5% level for 2nd vertebrae and 1% level for 3rd to 5th vertebrae). There was also a significant (at 1% level when compared to the control group) dose related incidence of non-ossified cervical centra (3rd vertebrae) observed in the 40 mg/kg dose group. It should be noted that the incidences for the 3rd and 4th vertebrae were outside the historical

control range for both groups and the incidence for the 5th vertebrae was outside the historical control range for the 100 mg/kg/day group only. The incidence of partially ossified parietals was reduced at 100 mg/kg/day compared to the concurrent controls but the reduction was not statistically significant when compared to the control group and was similar to historical control values.

Variants

There were no treatment-related effects on the incidence of external/visceral variants. A statistically significant increase in the incidence of slightly dilated ureters was seen at 10 mg/kg/day; however, this was within the recent historical control incidence and was not part of a dose-related trend.

Skeletal variants were seen in most foetuses. The incidence of non-ossified odontoid was higher in the 40 mg/kg/day groups (statistically significant at the 5% level) and 100 mg/kg/day group (statistically significant at the 5% level). The incidence of partially ossified transverse processes of the 4th lumbar vertebrae was higher across all treated groups (statistically significant at the 5% level for the 40 mg/kg dose group), although these incidences were outside historical control range and they did not form any coherent dose response. The incidence of fully ossified transverse processes of the 4th lumbar vertebrae was reduced at 100 mg/kg/day and to a lesser extent at 40 mg/kg/day. However, when the incidences of fully and partially ossified transverse processes were considered together, there was a slight increase in the 10 and 40 mg/kg/day groups and a slight decrease in the 100 mg/kg/day groups indicating that any effect was confined to the 100 mg/kg/day group. At 100 mg/kg/day there was an increased incidence of non-ossified calcaneum (statistically significant at the 5% level) and a slight increase (not statistically significant) in partially ossified 5th sternebrae although these data were comparable to recent controls.

Manus and *Pes* Assessment

There were no statistically significant effects on mean *manus* and *pes* scores.

4.3 Other effects

Not applicable

4.4 Analysis of Dosing Solutions

Analysis of Dosing Solutions

The achieved concentrations of the active ingredient of [redacted] 1,2-benzisothiazol-3-(2*H*)-one (BIT), were found to be within 8% of nominal values.

The chemical stability of each dosing formulation was established over a 34-day analysis interval which is greater than the period over which the formulations were used in the study. No evidence of any degradation of BIT was seen over this period at the 10 mg/mL level. At the lower dose level of 1 mg/mL, a small reduction was seen and by Day 34 the value was 94.5% of the original analysed concentration.

This reduction is within the limits of experimental error and is considered not to be significant.

The homogeneity of the 1 and 10 mg/mL formulations was shown to be satisfactory and the results of these analyses also show that there were no difficulties with subdivision of the bulk preparation into aliquots.

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and methods

Groups of 24 female rats (Wistar-derived Alpk:APfSD) were dosed by gavage with 10, 40 or 100 mg [redacted] kg/day in 0.5% (w/v) HMPC in 0.1% (w/v) aqueous Polysorbate 80 from Days 7-16 (inclusive) of gestation which thus included the period of organogenesis. A control group of animals received the vehicle alone.

The animals were received from the Specific Pathogen Free (SPF) colony, maintained at the [redacted] on the day of confirmation of mating (designated Day 1 of gestation). Clinical observations were made on arrival of the animals and subsequently daily throughout the study. Food consumption was also monitored throughout the study and the bodyweight of each animal was recorded on Days 1 and 4; daily from Days 7-16 (inclusive); and on Days 19 and 22 of gestation.

On Day 22 of gestation the females were sacrificed and macroscopic examination of all tissues was performed at *post mortem*. Uterine content and condition of the ovaries was examined. The number of live foetuses and intra-uterine deaths were recorded and the foetuses were weighed, examined for external and visceral abnormalities, sexed, eviscerated and stained for skeletal examination. The sex ratio was calculated and the abnormalities were classified as major, minor or variant (common observations that are not normally deleterious).

5.2 Results and discussion

There was no evidence of disease or infection amongst the animals. Analysis of the dosing formulations showed that the concentrations of the active substance of [redacted] (BIT) in 0.5% (w/v) HMPC in 0.1% (w/v) Polysorbate 80 were within acceptable limits and that the chemical stability of BIT in this vehicle during the dosing period was satisfactory.

At the top dose of 100 mg/kg/day, there was an increase in the incidence of abnormal respiratory noise, reduced bodyweight gain during the dosing period, reduced food consumption and an increase in stomach lesions at *post mortem* examination. Similar but less marked effects (except stomach lesions) were seen at 40 mg/kg/day. The bodyweight and food consumption data indicate that some individual animals were more susceptible to the effects of [redacted] at 40 or 100 mg/kg/day than the others. There were no treatment-related effects at 10 mg/kg/day. The moderate maternal toxicity at 100 mg/kg/day indicates that this dose was suitable for an assessment of teratogenicity.

There were no adverse effects on gravid uterus weight, litter weight, numbers of *corpora lutea* or numbers of intra-uterine deaths at any dose level. At 100 mg/kg/day, there was a small but statistically significant reduction in mean foetal weight.

Major defects were seen in 10 treated foetuses. There was no evidence to suggest that the type or distribution of these abnormalities was related to treatment.

The incidence of minor external/visceral defects was not affected by treatment. The overall incidence of skeletal defects was not significantly different from control in the treated groups. However, some individual defects, non-ossified cervical centra and partially ossified parietals showed statistically significant changes in incidence at 40 or 100 mg/kg/day. In addition, there were some individual skeletal variants which showed a change in incidence at 40 or 100 mg/kg/day.

Taken in conjunction with the slight reduction in foetal weight at 100 mg/kg/day, these results indicate a marginal effect on ossification (and hence foetotoxicity) at this dose level. At 40 mg/kg/day, the changes are considered to be too minor and too inconsistent to be of toxicological significance. This is substantiated by the absence of any effect on *manus* and *pes* scores, which are generally sensitive indicators of effects on ossification.

There were no indications of any effect at 10 mg/kg/day.

5.3 Conclusion

5.3.1	LO(A)EL maternal toxic effects	40 mg/kg/day Abnormal respiratory noise. Reduced body weight gain during dosing and slightly reduced food consumption.
5.3.2	NO(A)EL maternal toxic effects	10 mg/kg/day
5.3.3	LO(A)EL embryo toxic / teratogenic effects	100 mg/kg/day Small but statistically significant reduction in mean foetal weight. Marginal effect on ossification.
5.3.4	NO(A)EL embryo toxic / teratogenic effects	40 mg/kg/day
5.3.5	Reliability	2
5.3.6	Deficiencies	Yes, the degree of resorption was not described. The only evidence for a treatment-related effect on litter data parameters was 3.6% reduction in mean foetal weight for the 100

mg/kg/day group (this reduction was of statistical significance when compared to the control group, at the 5% level). This was exaggerated by one litter (from female 96) with a mean foetal weight of 3.7g. [Female 96 gained only 52 g during gestation (compared with the group mean of 128 g) despite having a litter of 13 foetuses and may have shown a high individual susceptibility to the toxicity of [redacted]

In this context, the lack of a description of the degree of resorption is not considered critical.

Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*October 2008*
Materials and Methods	*Applicant's version is accepted.*
Results and discussion	*Applicant's version is accepted.*
Conclusion	*Applicant's conclusions are adopted.*
Reliability	*1*
Acceptability	*Acceptable*
Remarks	*The fact described in second paragraph of deficiencies might not be considered a deficiency by itself. It is just an anomalous result arising from data obtained form only one animal.*

Table A6_8_1-1: Intergroup Comparison of Maternal Body Weights (g)

Period (Days)	Dose Level of [redacted] (mg/kg/day)				Approximate 95% Confidence Limit
	0 (Control)	10	40	100	
Initial Weight (Day 1)	251.5	252.1	255.8	254.9	-
Pre-dosing (1-7)	35.8	32.9	33.9	34.9	±2.7
1-4	20.8	19.7	20.4	21.6	±2.2
4-7	15.0	13.2	13.5	13.3	±1.5
During Dosing (7-16)	47.0	49.9	40.4	35.4**	±5.2
7-10	12.9	14.1	10.8	10.1	±2.2
10-13	16.9	16.7	15.8	9.4**	±2.8
13-16	17.2	19.1	13.8	15.9	±3.6
Post Dosing (16-22)	56.5	57.1	53.4	57.1	±5.4
16-19	32.6	33.4	36.2	29.6	±3.6
19-22	24.0	23.7	17.3	27.6	±5.6
Overall (1-22)	139.3	139.9	127.7	127.4	±8.8

** Statistically significant difference from the control at the 1% level.

Table A6_8_1-2: Intergroup Comparison of Maternal Food Comsumption (g/day)

Period (Days)	Dose Level of [redacted] (mg/kg/day)				Approximate 95% Confidence Limit
	0 (Control)	10	40	100	
Pre-dosing (1-7)	23.2	23.1 (22)[1]	24.2 (22)[1]	24.1	±0.9
1-4	21.8	21.6	22.5	22.6	±1.0
4-7	24.7	24.6 (22)[1]	25.5 (22)[1]	25.5	±0.8
During Dosing (7-16)	28.4	28.5 (22)[1]	27.5 (22)[1]	26.1**	±1.2
7-10	26.6	26.4 (22)[1]	26.3 (22)[1]	26.1	±1.1
10-13	28.0	28.4	27.7	25.2**	±1.3
13-16	30.5	30.5	28.4	26.8**	±1.5
Post Dosing (16-22)	29.0	28.7	27.0	27.3	±1.7
16-19	32.0	31.9	30.5	28.7**	±1.7
19-22	25.9	25.5	23.5	25.8	±2.3

[1] Mean calculated from a total of 22 animals
** Statistically significant difference from the control at the 1% level.

Table A6_8_1-3 -Maternal Macroscopic Findings *Post Mortem*

Description of Findings[1]	Dose Level of [redacted] (mg/kg/day)			
	0 (Control)	10	40	100
Number of females examined at termination	24	24	24	23[2]
Liver: prominent reticular pattern	2	1	1	1
Left Ovary: cystic bursa.	0	1	0	1
Kidneys: pale.	0	2	0	0
Right Kidney: slight pelvic dilatation	1	1	2	0
Right Kidney: moderate pelvic dilatation	1	1	1	0
Right Kidney: extreme pelvic dilatation	1	0	1	2
Left Kidney : moderate pelvic dilatation	0	0	1	0
Stomach: distended with gas	0	1	0	0
Stomach: raised lesions on surface of non-glandular region	0	0	0	3
Stomach: occasional brown, pitted areas on glandular mucosa	0	0	0	1
Small Intestine: contents yellow, 'lumpy and semi-solid	1	1	0	1
Caecum: gaseous	0	0	0	1
Colon/Rectum: gaseous	0	0	0	1
Number of females with findings	5	6	5	7

[1] The number of females exhibiting each symptom is presented
[2] One animal (number 91) from the 100 mg/kg/day group was found dead on Day 17. The following observations were made: snout: stained red; mammary tissue: red and inflamed; lungs: deep red blotches on all lobes, hole present in left lobe surrounded by clotted blood; stomach: raised lesions on entire surface, several haemorrhagic and ulcerated areas on the non-glandular area, contents gaseous with green, oily, viscous fluid; small and large intestines: extremely gaseous; left kidney: raised fatty cyst on ventral surface.

Table A6_8_1-4: Table for Teratogenic effects (Maternal effects)

Parameter		Dose Level of [redacted] (mg/kg/day)				Dose Response (+ / -)
		0 (Control)	10	40	100	
Number of dams examined		24	231	24	23	NA
Clinical findings during application of test substance				Abnormal Respiratory Noise 2	Abnormal Respiratory Noise 2	+
Mortality of dams (%)		0	0	0	4.2 3	-
Abortions		#	#	#	#	#
Body weight gain (g)	Day 1-7 (Pre dosing)	35.8	32.9	33.9	34.9	NA
	Day 7-16 (Dosing Period)	47.0	49.9	40.4	35.4	+
	Day 16-22 (Post dosing)	56.5	57.1	53.4	57.1	NA
	Day 1-22 (Overall)	139.3	139.9	127.7	127.4	+
Food Consumption (g/day)	Day 1-7 (Pre dosing)	23.2	23.1 (22)4	24.2 (22) 4	24.1	NA
	Day 7-16 (Dosing Period)	28.4	28.5 (22) 4	27.5 (22) 4	26.1**	+
	Day 16-22 (Post dosing)	29.0	28.7	27.0	27.3	NA
Water consumption		NA	NA	NA	NA	NA
Percentage of Animals with live Foetuses in utero at Termination		100	95.8%	100	1005	-
Necropsy findings in dams dead before end of test		-	-	-	Evidence of misdosing into the lung6	-

[1] 1 animal excluded from calculations since no live offspring were produced
[2] There was a dose-related increase (both in incidence of observations and numbers of animals affected) in abnormal respiratory noise in the 40 and 100 mg/kg/day groups. Otherwise clinical findings were of a type commonly found in Alpk:APfSD rats and the incidence was not affected by treatment with [redacted]
[3] One animal (number 91) from the 100 mg/kg/day group was found dead on Day 17
[4] Food consumption for 22 animals included in the mean value
[5] 100% of the 23 surviving females
[6] The animal dosed at 100 mg/kg/day found dead on Day 17 showed evidence of misdosing into the lung. This animal also had stomach lesions.
** Statistically significant difference from the control at the 1% level.
NA = Not applicable
# = Data not reported

Table A6_8_1-5: Teratogenic effects- Litter response (Caesarean section data)

Parameter	Dose Level of [redacted] (mg/kg/day)				dose-response + / -
	0 (Control)	10	40	100	
Mean Number of Corpora lutea	13.3	13.7	13.7	13.5	-
Mean Number of Implantations	12.3	13.0	12.8	12.2	-
Resorptions	#	#	#	#	#
Total number of litters	24	23	24	23	-
Total number of live foetuses	281	276	300	281	-
Total number of dead foetuses	14	21	6	5	-
Pre-implantation loss (Percentage)	7.8	5.4	7.0	8.0	-
Post-implantation loss (Percentage)	4.7	7.1	2.0	1.7	-
Mean number of foetuses/litter	11.7	12.1	12.5	12.0	-
Mean foetus weight (g)	4.97	5.04	4.90	4.79	+
Foetal sex ratio (% of male foetuses)	49.5	49.6	55.3	46.3	-
Mean Gravid Uterus Weight (g)	84.3	86.9	87.2	82.6	-
Placenta weight (mean)	#	#	#	#	#
Crown-rump length (mean)	#	#	#	#	#

Data not reported

Table A6_8_1-6: Table for Teratogenic Effects Examination of the foetuses

Parameter		Dose Level of [redacted] (mg/kg/day)				dose-response + / -
		0 (control)	10	40	100	
Number of External Malformations and Visceral Defects (Percentage)	Major	0 (0)	3 (1.1)	2 (0.7)	2 (0.7)	-
	Minor	38 (13.5)	26 (9.4)	28 (9.3)	21 (7.5)	-
	Variants	65 (23.1)	78 (28.3)	85 (28.3)	61 (21.7)	-
Number of Skeletal malformations (Percentage)	Major	0 (0)	0 (0)	2 (0.7)	2 (0.7)	-
	Minor	91 (32.4)	63 (22.8)	113 (37.7)	105 (37.4)	-
	Variants	266 (94.7)	267(96.7)	294* (98.0)	269 (95.7)	-

* Statistically significant difference from the control at the 5% level.

Table A6_8_1-7 –Summary of the Type and Incidence of Major Defects

Description of Findings[1]	Dose Level of [redacted] (mg/kg/day)			
	0 (Control)	10	40	100
External/Visceral				
Abdoment: ascites	0	1	1	1
Liver:cysts attached	0	1	0	0
Torso: *situs inversus totalis*	0	1	0	0
Brain : lateral ventricles moderately dilated.	0	1	0	0
Cleft lip, anophthalrnia (left)	0	0	0	1
Skeletal				
Fused mandibles.	0	0	0	1
Major vertebral defect.	0	0	1	1
Multiple minor defects of limbs and ribs.	0	0	1	0

Section A6	**Toxicological and Metabolic Studies**	
Subsection A6.8.1	**Teratogenicity test**	
Ann IIA, VI. 6.8.1		
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ]	**Technically not feasible [ ]** **Scientifically unjustified [X]**	
Limited exposure [ ]	**Other justification [ ]**	
Detailed justification:	Teratogenicity tests tests should normally be performed in the rabbit and one rodent species. In case that one study is performed the preferred species is the rabbit. The BIT dossier on submission contains a teratogenicity study in the rat, and this justification for non-submission of data is presented for the second species, the rabbit. This justification for non-submission of data is a brief outline of the detailed discussion presented in the following Expert Report; [redacted] 2007; Evaluation of Data on Teratogenicity of 1,2-Benzisothiazolin-3-one (BIT) with Respect to the Necessity of a Teratogenicity Study in Rabbits; Report. The full report is presented in the BIT Dossier IVA6.8.1/2. BIT showed no teratogenic effects in two experimental studies in rats, In addition, BIT caused no developmental toxic effects in a two-generation reproduction toxicity study in rats. Another three structurally similar isothiazolinones caused no teratogenic effects in two teratogenicity studies in rats and three teratogenicity studies in rabbits. In line with an observation valid for many classes of chemicals, slight embryotoxicity was observed for all isothiazolinones at doses leading to maternal toxicity. The NOELs for maternal and fetal toxicity in the studies performed with BIT were higher than the NOEL in a subchronic oral study in dogs. It should be noted that upon oral administration of BIT, as well as of the other isothiazolinones, the most sensitive toxicological endpoint of maternal toxicity was local forestomach irritation at the portal-of-entry, which is most likely caused by the chemical reactivity of the isothiazolinones moiety. It might be argued that this local effect may have been dose limiting in the teratogenicity studies and thus may have limited the ability to detect teratogenic effects. However, the dose-response relationship for forestomach lesions was very similar after gavage dosing of BIT in the teratogenicity study and after administration of BIT via the diet in the 2-generation study. At a dose level of about 50 mg/kg/day, slight forestomach lesions were reported. The effect severity increased at higher doses, while it was not observed any more at lower doses. From this it may be concluded that the gavage dosing technique was not dose limiting in the teratogenicity studies. In addition, all isothiazolinones are classified as severe skin and eye irritants or as corrosive. Therefore, other routes of administration, such as dermal application or inhalation, cannot be employed in teratogenicity studies. Taken together, the portal-of-entry effects of BIT and the other isothiazolinones are considered not to	

Section A6	Toxicological and Metabolic Studies	
Subsection A6.8.1 **Ann IIA, VI. 6.8.1**	**Teratogenicity test**	
	have limited or compromised the identification of a teratogenic hazard. Based on these results it can be concluded that the isothiazolinones as a class lack the potential to cause developmental toxicity and, thus, that sufficient information is available to complete a risk assessment of BIT without generating teratogenicity data in a second species (rabbit). This conclusion is supported by the qualitatively and quantitatively similar toxicological profile for BIT and other isothiazolinones, including the observations that they produce minor effects upon repeated exposure and lack genotoxic and carcinogenic potential. An additional teratogenicity study would be imprudent in that it would result in the needless sacrifice of laboratory animals. In fact, in Chapter 1 of the data requirements for biocidal product types it is stated that the amount of animal testing, especially on vertebrates, shall be minimised and that all unnecessary testing of substances and preparations must be avoided (Council Directive 98/8/EC, 2000). **Summary** Non-inclusion of this data is justified on the basis that sufficient information is available to complete a risk assessment of BIT without generating teratogenicity data in a second species (rabbit).	
Undertaking of intended data submission [ ]		
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*October 2008.*	
Evaluation of applicant's justification	*Applicant's justification is accepted.*	
Conclusion	*Applicant is exempted to perform teratogenicity study in rabbits.*	
Remarks		

Section A6 Toxicological and Metabolic Studies

Subsection A6.8.2 Multigeneration Reproduction Toxicity Study

Annex Point IIA VI.6.8.2 ***Rat Feeding Study***

			Official use only
		1 REFERENCE	
1.1	**Reference**	[redacted] 2002; [redacted] (BIT): Two Generation Oral (Dietary Administration) Reproduction Toxicity Study in the Rat. [redacted] Report No. 1803/14-D6154.	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Arch Chemicals Inc	
1.2.2	Company with letter of access	Clariant Production UK Ltd and Thor GmbH	
1.2.3	Criteria for data protection	Data on existing substance for first entry in to Annex I	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	Yes EPA Health Effects Test Guidelines OPPTS 870.3800 (1998)	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	Batch Numbers [redacted]	
3.1.2	Specification	As given in section 2	
3.1.2.1	Purity	Batch No. [redacted]: 93.1% Batch No. [redacted] 92.7 ± 1.1%	
3.1.2.2	Stability	The test material was stated to be stable by the Sponsor. The expiry dates stated by the Sponsor were: Batch No. [redacted]: 19 October 2002 Batch No. [redacted]: 30 January 2003	
3.2	**Test Animals**		

Section A6 Toxicological and Metabolic Studies

Subsection A6.8.2 Multigeneration Reproduction Toxicity Study

Annex Point IIA VI.6.8.2 ***Rat Feeding Study***

3.2.1	Species	Rat
3.2.2	Strain	Crl:WI(Glx/BRL/Han)BR
3.2.3	Source	[redacted]
3.2.4	Sex	Male and female
3.2.5	Age/weight at study initiation	At the start of treatment, P generation animals were approximately 7 to 9 weeks old and were within the weight range 172.9 to 227.3 g for males and 127.5 to 175.5 g for females.
3.2.6	Number of animals per group	P generation: 24 male and 24 female F_1 generation: 24 male and 24 female
3.2.7	Mating	After the growth/maturation phases of the P and F_1 animals, one male was housed with one female from the same treatment group for up to 15 days. Mating was confirmed by the presence of a vaginal plug *in situ* or sperm in a vaginal washing. On confirmation of mating, vaginal washing was discontinued and the male was re-housed. The day on which mating was confirmed was designated Day 0 of gestation.
3.2.8	Duration of mating	Up to 15 days
3.2.9	Deviations from standard protocol	Not applicable
3.2.10	Control animals	Yes
3.3	**Administration/ Exposure**	Oral
3.3.1	Animal assignment to dosage groups	P generation: assigned to treatment groups during the acclimatisation period using a randomisation procedure based on stratified body weight. F_1 generation: at weaning, 24 males and 24 females from the available litters were randomly selected.
3.3.2	Duration of exposure before mating	10 weeks for P generation and F_1 generation animals. Both the P and F_1 generation animals underwent 10 weeks of maturation, whilst receiving the control or test article formulations prior to pairing.
3.3.3	Duration of exposure in general P, F1, F2 males, females	P generation (females): Maximum of 18 weeks P generation (males): Maximum of 19 weeks

F_1 generation (females): Maximum of 19 weeks

F_1 generation (males): Maximum of 21 weeks

Refer to Table A6_8_2-1 for the dates and phases of exposure.

Oral

3.3.4 Type

Dietary

3.3.5 Doses

The group mean achieved intakes of [redacted]

Generation	[redacted] Dose Level (ppm)	Intake mg/kg/day	
		Males	Females
P	250	18.5	27.0
	500	37.2	54.2
	1000	75.1	112.0
F_1	250	24.0	28.2
	500	48.0	56.6
	1000	97.8	114.8

Compound Consumption (mg/kg/day) = (Diet Concentration (ppm) × food intake (g/day)) / mid interval body weight (g)

3.3.6 Vehicle

The test item was added directly to diet.

3.3.7 Concentration in vehicle

0 (control), 250, 500 and 1000 ppm [redacted] in diet.

Test diets were prepared weekly.

Analysis of Dietary Formulations

The stability, homogeneity and achieved concentration of selected samples of dietary formulation were determined using a validated HPLC UV method.

Stability and Homogeneity

Dietary formulations prepared at 250 and 1000 ppm were found to be homogenous with coefficients of variation of < 6% at 250 and 1000 ppm (two replicates, sampled from the top, middle and bottom of the prepared diets were analysed).

Diets (prepared using [redacted]) were analysed in triplicate after storage at room temperature (10 to 30 °C)

		for 7 and 10 days. There was no evidence of decrease in test article concentration during the 10 day storage period at room temperature <u>Achieved Concentrations</u> Samples were taken from the dietary formulations prepared for Week 1 (start of treatment) and Weeks 17, 19, 34, 36, 37 and 38 and were analysed to determine the achieved concentration of [redacted] Analysis of samples from the diets prepared for administration in Weeks 1, 17 and 19 of the study showed that the achieved concentrations were within the target range. All values were within the range 89 to 110% of nominal. Analysis of samples from the diets prepared for administration in Weeks 34, 36, 37 and 38 of the study showed that the achieved concentrations were below the target range. Test article [redacted] was used for the formulations prepared in Weeks 1, 17 and 19, whereas [redacted] was used for the preparation of diets from Week 32. It was noted that during the analyses of the diets using Batch [redacted] for confirmation of homogeneity, the achieved concentrations were also low. The measured concentrations of [redacted] in the diet administered to the second generation at weeks 34, 36, 37, and 38 were low. As a percentage of nominal, the test diet concentrations ranged from 60-79% at Week 34, 38-56% at Week 36, 47-81% at Week 37, and 56-61% at Week 38. Examination of the diet preparation records verified that the diets had been accurately prepared on all occasions. The report author attributed the anomolous results to an unknown property, possibly diet-binding, and not to incorrect dietary concentrations. Hyperplasia of the stomach was noted in the mid- and high-dose groups of both test article batches, and decreased body weight was also noted in the mid- and high-dose groups. These observations are consistent with the effects of the test article and therefore the later analytical data are not considered to reflect the true dose rate. Also the perceived deficiency in test article concentration, due to occurring late in the dosing regimen, would in any case have been unlikely to affect the reproductive outcome of the study. Accordingly the anomalous analytical results are not considered to have affected the integrity or outcome of the study.
3.3.8	Total volume applied	Not applicable
3.3.9	Controls	Plain diet

Section A6 Toxicological and Metabolic Studies

Subsection A6.8.2 Multigeneration Reproduction Toxicity Study

Annex Point IIA VI.6.8.2 ***Rat Feeding Study***

3.4	**Examinations**	
3.4.1	Clinical signs and mortality	Clinical Signs All animals were examined at least once daily for signs of ill health or overt toxicity. Any abnormalities of appearance or behaviour or other signs of reaction to treatment Morbidity and mortality All animals were examined twice daily to detect any which were dead or moribund. Moribund animals were killed by an intraperitoneal injection of sodium pentobarbitone solution and examined macroscopically.
3.4.2	Body weight	Individual body weights were recorded weekly for the males. For the females, individual body weights were recorded weekly during the growth/maturation phases until confirmation of mating, on Days 0, 7, 14 and 20 of gestation and on Days 1, 4, 7, 14 and 21 *post-partum*.
3.4.3	Food/water consumption	Diet and water Throughout the study the animals had access *ad libitum* to SQC Rat and Mouse Breeder Diet No 3, Expanded, Ground Fine (Special Diets Services Ltd, Witham). Mains water was available *ad libitum* from an automatic watering system (group housed animals) or from glass water bottles attached to the cages (single housed animals). The contents of the bottles were changed daily. Food Intake The food consumed by each cage of animals was determined weekly during the pre-pairing periods (males and females). Individual food intake of females was recorded for Days 0 to 3, 3 to 7, 7 to 10, 10 to 14, 14 to 17 and 17 to 20 of gestation (mated females) and for Days 1 to 4, 4 to 7, 7 to 10, 10 to 14, 14 to 17, 17 to 19 and 19 to 21 *post-partum* (littering females) but reported on the body weight intervals.
3.4.4	Oestrus cycle and mating performance	Daily vaginal washings were taken and the stage of oestrus recorded for each female from 21 days prior to the start of the pairing period until confirmation of mating or the end of the 15 day pairing period in each generation.
3.4.5	Duration of gestation	The following sperm parameters were evaluated in P and F_1 parental animals. Testis weight (All males) Epididymis (total and left cauda) weight (All males)

		Enumeration of cauda epididymal sperm reserve (All males) Sperm motility (All males) Sperm morphology (Control and high group only) Enumeration of homogenisation-resistant spermatids in testes and epididymis (the left testis from all males was frozen in CO_2 and stored at -20°C pending possible enumeration of homogenisation-resistant spermatids).
3.4.6	Sperm parameters	<u>P and F_1 Litter Data</u> All females were allowed to litter and the date of parturition and the duration of gestation were recorded. The following data were recorded for each litter to Day 21 *post-partum*: • Number of pups born (live and dead) • Daily live litter size and sex (reported on Days 1, 4, 7, 14 and 21) • Daily clinical observations • Individual pup weights on Days 1,4, 7, 14 and 21 *post-partum* • Necropsy findings of dead and culled pups where condition permitted On Day 4 *post-partum*, litters were culled to a maximum of eight pups with an equal sex distribution where possible. Animals considered unlikely to survive to weaning were pre-selected for cull and a random selection procedure was used for additional pups. <u>Weaning of the F_{1a} Offspring</u> On Day 21, 24 pups/sex were randomly selected from available litters for the F_1 generation. Pups not selected were killed and necropsied. <u>Maturation phase F_1 generation</u> The selected F_1 animals underwent 10 weeks of maturation, whilst receiving the control or test article formulations prior to pairing. Vaginal opening (females) and balano-preputial separation (males) were assessed daily from 30 or 40 days of age, respectively, until development was complete.
3.4.7	Offspring	The following organs, as appropriate, from all P and F_1 parental animals were dissected free from fat and other contiguous tissue and weighed before fixation: Adrenals, brain, epididymides (total and left cauda), kidneys, liver, ovaries, pituitary, prostate, seminal vesicles (with coagulating gland), spleen, target organs (stomach #), testes and uterus (with oviducts and cervix).

		# The stomach was not weighed since animals not starved prior to necropsy.
3.4.8	Schedule of sacrifice	Samples of the following tissues from all P and F_1 parental animals were fixed in relevant preservatives: Adrenal glands, one testis, one epididymis, seminal vesicles, prostate, coagulating gland, pituitary, ovaries, uterus (with oviducts and cervix), vagina, target organs (stomach), lesions (plus corresponding control). The right testis and epididymis were preserved in Bouin's fluid and embedded in paraffin wax. All other tissues were preserved in 10% neutral buffered formalin. Grossly abnormal tissue and target organs of the weanling F_1 and F_2 pups selected for macroscopic examinations were similarly retained. Samples of the above tissues from the first ten animals of each sex from the control and high dose groups, reproductive organs from all apparently infertile animals and all treatment-related lesions were embedded in paraffin wax, sectioned at a nominal thickness of 5µm and stained with haematoxylin and eosin. For the F_1 females, a quantitative evaluation of primordial follicles was conducted on ten ovarian sections, at 100 µm apart, from the inner third of each ovary. Examination included enumeration of the total number of primordial follicles from these twenty sections for comparison with control values. All sections were examined by the study pathologist using light microscopy.
3.4.9	Necropsy	F_1 pups not selected for mating, (F_{1a}) were killed after weaning. All of these pups, F_1 pups found dead and culled pups were examined only macroscopically for structural or pathological changes. Three pups per sex from each litter of F_2 pups surviving to weaning were similarly examined. The remaining F_2 pups were discarded. The brain, spleen and thymus from one randomly selected pup per sex per litter of unselected F_1 pups and of surviving F_2 pups were similarly processed and weighed. Particular attention was paid to the reproductive organs of pups examined at weaning.
3.5	**Statistics**	<u>Data processing</u> Data were processed, where appropriate, to give litter mean values, group mean values and standard deviations. Some tables and appendices presented in the report are computer generated. The group mean and individual data are generated independently from the values held on a data base and rounded

appropriately for inclusion in the report. As a consequence calculation of group mean data from the individual data presented in the report will, in some instances, yield a minor variation in the last significant figure.

Calculations

Group mean values for certain litter parameters were calculated as follows:

Mean 1: includes data from all surviving females giving birth including those showing total litter loss post-partum.

Mean 2: includes data from females rearing some young to weaning.

A number of indices were used, where appropriate, to evaluate reproductive function: These included:

Mating index

Female fecundity index

Male fecundity index

Female fertility index

Male fertility index

Statistical Evaluation

Body weight, body weight gains, necropsy body weights, food consumption and seminology variables (both P and F1 generations) were analysed using one-way analysis of variance (ANOVA). Levene's test for equality of variances among the groups was performed. Where this showed no evidence of heterogeneity ($P \geq 0.01$), pairwise comparisons with control were made using Dunnett's test. A regression test was performed to determine whether there was a relationship between increasing dose and response. A significant trend ($P < 0.05$) was only reported where none of the pairwise comparisons was significant.

Where Levene's test showed evidence of heterogeneity ($P < 0.01$), the data were analysed using the same methods after applying a log-transformation.

Seminology and ovarian follicle data were analysed using the two-sample t-test where data for only two groups was available.

The number of implantation sites, number of pups born, percentage of male pups Day 1 and pup weights (both P and F_1 generations) and physical development data (F_1 generation) were analysed using non-parametric methods. The non-parametric methods employed were the Kruskal-Wallis ANOVA, the Terpstra-Jonckheere test for a dose related trend and the Wilcoxon rank sum test for pairwise comparisons. Where the Kruskal-Wallis ANOVA was not significant, the pairwise comparisons were not reported in order to protect the Type I error.

The proportions of females with post-implantation survival index, live birth index and viability indices of 100% and gestation, mating, fertility and fecundity indices (both P and F_1 generations) were analysed using the Cochran-Armitage test for dose-response and Fisher's exact test for pairwise comparisons. The tests were interpreted with one-sided risk for decreased incidence with increasing dose.

Organ weights were analysed using Analysis of Covariance (ANCOVA) and Dunnet's test, for each sex separately, using the necropsy body weight as covariate. This analysis depends on the assumption that the relationship between the organ weights and the covariate is the same for all groups, and the validity of this assumption was tested. Levene's test for equality of variances across the groups was also performed for all organ weights and where this showed evidence of heterogeneity (male pituitary and female uterus weights, $P < 0.0l$) the organ was analysed using one-way ANOVA on absolute organ weights and organ to necropsy body weight ratios.

4 RESULTS AND DISCUSSION

4.1 Effects

4.1.1 Parent males

Morbidity and Mortality

One male in the high dose group was found dead at the beginning of Week 8 of the study due to the presence of a large subcutaneous mass in the axillary region, swelling and blue colouration of the hind leg and general poor condition. Necropsy examination showed that in addition to the mass, this animal had enlarged hind leg muscle, abnormal contents of the oesophagus, blood in the thoracic cavity and markedly dark lungs.

All other animals survived to the scheduled kill.

Clinical Observations

The nature and intergroup distribution of the clinical observations made for parent males did not indicate any dose related effects.

Body Weights

There were no adverse effects of treatment on group mean body weight gain observed in males.

Food Intake

Group mean food intake was similar in all male groups throughout the study.

Mating

The majority of animals mated during the first oestrous cycle. In all groups, the pre-coital time was 2 to 2.5 days and the mating, fertility and fecundity indices were similar in all groups.

One control, one low dose and two intermediate dose group males failed to sire pregnancies after positive evidence of mating. At necropsy examination, the low dose male had abnormal epididymides and testes and one of the intermediate dose males had a small prostate and small seminal vesicles. The reproductive organs of the other two males were macroscopically normal. None of these findings are considered to have any toxicological significance.

Seminology

Spermatogenesis, as assessed by measurements of sperm motility, count and morphology, was unaffected by treatment.

Organ Weights

In the high dose group, the mean liver weight of the males was higher than that of the controls and the difference was statistically significant when the weight was adjusted for the mean terminal body weight ($P < 0.01$, Dunnett's test). However, female liver weight was unaffected by treatment and therefore the slight increase in male liver weight is not considered to represent an adverse effect of treatment. Similarly, although there was a slight, dose-related reduction in adjusted testes weight that was significant in the high dose group ($P < 0.05$, Dunnett's test), in the absence of any effects on mating or seminology data, this too is not considered to be an adverse effect of treatment.

All other organ weights were unaffected by treatment.

Necropsy and Histopathology

There were no treatment-related effects noted at necropsy examination of the males.

Microscopic findings in the reproductive organs and related tissues were comparable in all groups. In the stomach of the intermediate and high dose group animals, limiting ridge hyperplasia was noted in 5/24 intermediate dose males and 16/23 high dose males. The finding was characterised by a minor increase in the thickness of the epithelium at the limiting ridge between the forestomach and fundus, with variable rete peg formation and folding of the epithelium.

4.1.2 Parent females

Morbidity and Mortality

All parent females survived to the scheduled kill.

Clinical Observations

The nature and intergroup distribution of the clinical observations made for parent females did not indicate any dose related effects.

Body Weights

There were no adverse effects of treatment on group mean body weight gain in females during the pre-pairing period, gestation or

lactation.

Food Intake

Group mean food intake was similar in all female groups throughout the study.

Mating

The majority of animals mated during the first oestrous cycle. In all groups, the pre-coital time was 2 to 2.5 days and the mating, fertility and fecundity indices were similar in all groups.

Organ Weights

There were no treatment related effects on female organ weights.

Necropsy and Hislopathology

In females, there were treatment-related incidences of macroscopic abnormalities of the stomach including raised foci, reddening and thickening. Other findings in females were comparable in all groups.

Microscopic findings in the reproductive organs and related tissues were comparable in all groups. In the stomach of the intermediate and high dose group animals, limiting ridge hyperplasia was noted in 8/24 intermediate dose females and 16/24 high dose females. The finding was characterised by a minor increase in the thickness of the epithelium at the limiting ridge between the forestomach and fundus, with variable rete peg formation and folding of the epithelium. In one intermediate and three high dose females there was also squamous cell hyperplasia of the forestomach and in one high dose female, forestomach gastritis.

Litter Data

One female from the high dose group showed total embryo-foetal loss and two, one and three females from the low, intermediate and high dose groups, respectively showed total litter loss. Subsequent necropsy examination of these animals showed no macroscopic findings indicative of an adverse effect of treatment.

The mean duration of gestation, mean numbers of implantations, numbers of pups born and pup survival, were similar in all groups. Pup sex ratio (percentage male pups) was slightly lower than expected in the high dose group, however these differences were not statistically significant and did not appear to be dose related.

The mean weight of the high dose pups on Day 1 *post partum* was slightly lower (6%) than that of the controls but the difference was not statistically significant. The mean weight gain of these offspring was also lower than that of the controls so that at Day 7 *post partum* the mean pup weight gain of the high dose group was 8% lower than that of the controls. However, after Day 7, the weight difference between the pups in the control and high dose groups was not so great and the overall mean pup weight gain over the lactation period

was similar in all groups.

At necropsy examination of the weanling offspring, there were no findings indicative of an adverse effect of treatment.

Group mean spleen and brain weights of the F_{1a} weanlings, both absolute and when adjusted for terminal body weight, were unaffected by treatment. However, mean adjusted thymus weight of the high dose males was significantly lower than control ($P < 0.05$, Dunnett's test), but since the thymus weight of the high dose females was unaffected by treatment the effect in the males is not considered to be related to treatment.

4.1.3 F_1 males

Morbidity and Mortality

There were no unscheduled male deaths.

Clinical Observations

The nature and intergroup distribution of the clinical observations made for F_1 males did not indicate any dose related effects.

Body Weights

Males in the high dose group gained slightly less weight than the controls during the whole study, but the differences from control were only significant between Weeks 15 and 19 ($P < 0.05$, Dunnett's test).

Food Intake

There were no adverse effects of treatment on group mean food intake in males at any time point during the F_1 phase of the study.

Physical Development

The mean day of balano-preputial separation of the intermediate and high dose group males was 45 days compared to 44 in the low dose group and 43 in the controls. The differences from control were statistically significant ($P < 0.05$, Wilcoxon rank sum test). However, the age range for balano-preputial separation in the intermediate and high dose groups was within the control range and the very slight lengthening of the time to separation probably reflects a marginal developmental delay since the males in these groups were slightly lighter than the controls at the start of the F_1 generation.

Mating data

The mating, fertility and fecundity indices were unaffected by treatment.

Seminology

Mean total sperm count in the high dose group was significantly higher than control ($P < 0.05$, Dunnett's test) whereas the velocity parameters (average path velocity, straight line velocity and

curvilinear velocity) in this group were slightly lower than control (significant result from dose response test for curvilinear velocity, P < 0.05). However, sperm motility, straightness and morphology were similar in all groups and it is not considered that spermatogenesis was adversely affected by treatment.

Organ Weights

Male organ weight data were similar in all groups.

Necropsy and Histopathology

At necropsy examination, there was increased incidences of raised foci and thickening of the stomachs (observed in males to a lesser extent than females). All other macroscopic findings were common in this strain and age of rat.

In the high dose group animals, microscopic findings in the reproductive organs and related tissues were comparable with controls. Limiting ridge hyperplasia of the stomach was noted in 6/24 intermediate dose males and 12/24 high dose males.

4.1.4 F_1 females

Morbidity and Mortality

One low dose group female was killed in Week 9 of the pre-pairing period following clinical observation of teeth abnormalities and a swollen head. Necropsy examination confirmed the clinical observations but showed no other macroscopic abnormalities.

All other females survived to the scheduled kill.

Clinical Observations

The nature and intergroup distribution of the clinical observations made for F_1 females did not indicate any dose related effects.

Body Weight

During the pre-pairing period, the high dose group females gained slightly less weight than the controls and the difference was statistically significant over the first five weeks (P < 0.05, Dunnett's test). Consequently, the females in the high dose group began the gestation period slightly lighter than the controls however there were no statistically significant dose related differences during the gestation and lactation periods.

Food Intake

There were no adverse effects of treatment on group mean food intake in females at any time point during the F_1 phase of the study.

Physical Development

There was no adverse effect of treatment on vaginal opening.

Mating data

All animals, except for one low dose group and one high dose group

female mated during the first oestrous cycle. In all groups the pre-coital time was 2.5 to 3 days. One control female and one in the intermediate dose group failed to mate despite being re-paired with a proven male. Necropsy and pathology examinations revealed no macroscopic or microscopic abnormalities in these females.

The mating, fertility and fecundity indices were unaffected by treatment.

Organ Weights

Female organ weight data were similar in all groups.

Necropsy and Histopathology

At necropsy examination, there were increased incidences of raised foci in, and thickening of, the stomachs of the high dose animals, particularly the females. All other macroscopic findings were common in this strain and age of rat.

In the high dose group animals, microscopic findings in the reproductive organs and related tissues were comparable with controls. In the intermediate and high dose groups, limiting ridge hyperplasia of the stomach was noted in 4/24 intermediate dose females and 17/24 high dose females. In the high dose females there was also squamous cell hyperplasia (14/24), forestomach gastritis (2/24), hyperkeratosis (7/24) and erosion/ulcer (1/24).

Follicle Evaluation

There was no statistically significant difference between the mean numbers of ovarian follicles in the control group and high dose group.

Litter Data

One female from the intermediate dose group showed total embryo-foetal loss and two females from the control and high dose groups and one from each of the low and intermediate dose groups showed total litter loss.

The mean duration of gestation was unaffected by treatment and there was no statistically significant difference in the pup sex ratio between dose groups. There was a dose-related reduction in mean numbers of implantation sites ($P < 0.05$, Terpstra-Jonkheere test) and a consequent dose-related reduction in mean numbers of pups born ($P < 0.05$, Terpstra-Jonkheere test).

4.1.5 F_2 males

More pups in the high dose group died between Days 1 and 4 *post partum* compared to the control group, resulting in a significantly lower dose-response in the viability index ($P < 0.05$, Cochran-Armitage test). However, pup survival between Day 4 *post partum* and weaning was unaffected by treatment.

The mean weight of the high dose group male pups on Day 1 *post partum* was slightly higher than control resulting in an increasing

dose-response (P < 0.05, Terpstra-Jonkheere test). This was probably due to the slightly smaller litter sizes in this group.

The mean pup weight gain in the treated male groups was lower than the control over the lactation period although the effect was not dose-related. The differences in the mean percentage weight change (calculated from male and female data) were statistically significant (P < 0.05 for the low and intermediate groups; P < 0.01 for the high dose group, Wilcoxon rank sum test)

The macroscopic findings at necropsy examination of the male weanling offspring were largely unremarkable and did not indicate any dose related effects.

There were no adverse effects of treatment on the spleen, brain or thymus weights of the selected male F_{2a} weanlings.

4.1.6 F_2 females

More pups in the high dose group died between Days 1 and 4 *post partum* compared to the control group, resulting in a significantly lower dose-response in the viability index (P < 0.05, Cochran-Armitage test). However, pup survival between Day 4 *post partum* and weaning was unaffected by treatment.

Mean pup weight gain in the treated female groups was lower than control over the lactation period although the effect was not dose-related. The differences in the mean percentage weight change (calculated from male and female data) were statistically significant (P < 0.05 for the low and intermediate groups; P < 0.01 for the high dose group, Wilcoxon rank sum test)

The macroscopic findings at necropsy examination of the female weanling offspring were largely unremarkable and did not indicate any dose related effects.

There were no adverse effects of treatment on the spleen, brain or thymus weights of the selected female F_{2a} weanlings.

4.2 Other

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and methods

The objective of the study was to investigate the effects of the test article, [redacted] (1,2-Benzisothiazolin-3-one, aka BIT), on the integrity and performance of the male and female reproductive systems including gonadal function, the oestrous cycle, mating behaviour, conception, pregnancy, parturition, lactation, weaning and the growth and development of the offspring when administered orally, by diet, to two successive generations. The study was designed to meet the known requirements of the EPA Health Effects Test Guidelines OPPTS 870.3800 (1998).

Groups of 24 male and 24 female parental rats (P generation) were given [redacted] by admixture with the diet at dose levels

of 250, 500 or 1000 ppm. A similar group received the control diet only. The animals received the test diet for 10 weeks before pairing for two weeks. The dosing continued during this pairing period and throughout the resulting pregnancies. The P generation females were allowed to litter and rear their offspring (F_{1a}) to weaning. Administration of the test article continued throughout the weaning of the F_1 offspring until necropsy.

Twenty-four animals of each sex were randomly selected from each group to form the filial (F_1) generation. Direct treatment of the F_1 generation continued during their maturation period (10 weeks), the mating period (up to two weeks) and throughout the resulting pregnancies and weaning of the F_2 offspring up until necropsy. All F_1 females were allowed to litter and rear their offspring (F_{2a}) to weaning.

The male and female intakes of [redacted] for each dose group are presented in the following table.

Generation	[redacted] Dose Level (ppm)	Intake mg/kg/day	
		Males	Females
P	250	18.5	27.0
	500	37.2	54.2
	1000	75.1	112.0
F_1	250	24.0	28.2
	500	48.0	56.6
	1000	97.8	114.8

5.2 Results and discussion

P Generation

Clinical observations, body weights and food intakes were unaffected by treatment.

Mating data, duration of gestation, numbers of implantations, numbers of pups born and pup survival were similar in all groups.

Mean pup weight gain of the high dose pups was slightly lower than control over the first week post partum. However, over the whole lactation period, mean pup weight gain was similar in all groups.

There were no adverse effects of treatment on the seminology data.

In the high dose group, mean liver weight of the males was slightly higher than, and mean testes weight slightly lower than, control. Neither of these findings was considered to represent adverse effects

of treatment.

Minor limiting ridge hyperplasia in the stomach was noted in some intermediate and many high dose animals. Squamous cell hyperplasia and forestomach gastritis were also seen in a few animals.

F_1 Generation

Males in the high dose group gained slightly less weight than the controls during the study and the high dose females gained slightly less weight during the pre-pairing period, only.

Clinical observations and food intakes were unaffected by treatment.

Physical development of the F_1 generation, mating data, duration of gestation and F_{2a} pup sex ratio were unaffected by treatment. Pup survival to Day 4 *post partum* and mean pup weight gain were slightly lower in the high dose group compared to control.

Seminology investigations, organ weights and ovarian follicle counts were unaffected by treatment.

In the intermediate and high dose groups, limiting ridge hyperplasia in the stomach was noted. This was most prominent in the high dose females where there was also squamous cell hyperplasia, forestomach gastritis, hyperkeratosis and erosion/ulcer.

5.3 Conclusion

Dietary administration of 1000 ppm [redacted] to rats for two generations produced slight adult toxicity in the F_1 generation in terms of lower body weight gain, and in both generations, limiting ridge hyperplasia of the stomach together with incidences of squamous cell hyperplasia, forestomach gastritis, keratosis and erosion/ulcer. At this concentration, the growth of the offspring was slightly impaired and in the F_{2a} offspring, there was a slight reduction in pup survival.

At the 500 ppm dose level there were incidences of limiting ridge hyperplasia in the stomach.

There were no adverse effects of treatment at 250 ppm, equivalent to an approximate overall mean intake of 24 mg/kg/day.

5.3.1 LO(A)EL

5.3.1.1	Parent males	LO(A)EL: 500 ppm (mean dose of 37.2 mg/kg/day) based on hyperplasia of the limiting ridge of the stomach
5.3.1.2	Parent females	LO(A)EL: 500 ppm (mean dose of 54.2 mg/kg/day) based on hyperplasia of the limiting ridge of the stomach
5.3.1.3	F_1 males	LO(A)EL: 1000 ppm (mean dose of 97.8 mg/kg/day) based on impaired growth and survival of pups

5.3.1.4	F_1 females	LO(A)EL: 1000 ppm (mean dose rate of 114.8 mg/kg/day) based on impaired growth and survival of pups
5.3.1.5	F_2 males	Not applicable: F_2 animals not dosed
5.3.1.6	F_2 females	Not applicable: F_2 animals not dosed
5.3.2	NO(A)EL	
5.3.2.1	Parent males	250 ppm (mean dose rate of 18.5 mg/kg/day)
5.3.2.2	Parent females	250 ppm (mean dose rate of 27.0 mg/kg/day)
5.3.2.3	F_1 males	500 ppm (mean dose rate of 48.0 mg/kg/day)
5.3.2.4	F_1 females	500 ppm (mean dose rate of 56.6 mg/kg/day)
5.3.2.5	F_2 males	Not applicable : F_2 animals not dosed
5.3.2.6	F_2 females	Not applicable : F_2 animals not dosed
5.3.3	Reliability	1
5.3.4	Deficiencies	Yes. The measured concentrations of [redacted] in the diet administered to the second generation at weeks 34, 36, 37, and 38 were low. As a percentage of nominal, the test diet concentrations ranged from 60-79% at Week 34, 38-56% at Week 36, 47-81% at Week 37, and 56-61% at Week 38. Examination of the diet preparation records verified that the diets had been accurately prepared on all occasions. The report author attributed the anomolous results to an unknown property, possibly diet-binding, and not to incorrect dietary concentrations. Hyperplasia of the stomach was noted in the mid- and high-dose groups of both test article batches, and decreased body weight was also noted in the mid- and high-dose groups. These observations are consistent with the effects of the test article and therefore the later analytical data are not considered to reflect the true dose rate. Also the perceived deficiency in test article concentration, due to occurring late in the dosing regimen, would in any case have been unlikely to affect the reproductive outcome of the study. Accordingly the anomalous analytical results are not considered to have affected the integrity or outcome of the study.

Section A6 Toxicological and Metabolic Studies

Subsection A6.8.2 Multigeneration Reproduction Toxicity Study

Annex Point IIA VI.6.8.2 ***Rat Feeding Study***

Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*October 2008*
Materials and Methods	*Applicant's version is accepted.*
Results and discussion	*Applicant's version is accepted.*
Conclusion	*Applicant's conclusion is adopted.*
Reliability	*1*
Acceptability	*Acceptable*
Remarks	*Minor non-relevant mismtaches with data of Doc. IV were detected in Table A6-8-2-3.*

Table A6_8_2-1: Study Plan

Week of Study	Dates	Phase	
1 to 10	30 Oct 2000 to 7 Jan 2001	P Generation	Maturation
11,12	8 Jan 2001 to 22 Jan 2001		Mating
14,15	31 Jan 2001 to 5 Feb 2001		Littering
17,18	21 Feb 2001 to 27 Feb 2001		Weaning F_{1a} and P generation female kills
19	5 Mar 2001 to 9 Mar 2001		P generation male kills
19 to 28	5 Mar 2001 to 13 May 2001	F_1 generation	Maturation
29,30	14 May 2001 to 28 May 2001		Mating
32,33	6 June 2001 to 15 June 2001		Littering
35,36	27 June 2001 to 7 Jul 2001		Weaning F_{2a} and F_1 generation female kills
38	16 Jul 2001 to 19 Jul 2001		F_1 generation male kills

Table A6_8_2-2: Summary of Adult Performance- P Generation

Male/Female Performance	Group 1	Group 2	Group 3	Group 4	Statistics
	Control	250 ppm [redacted]	500 ppm [redacted]	1000 ppm [redacted]	
Males					
Number in Group	24	24	24	24	
Number Died/Killed before Pairing	0	0	0	1	
Number Inducing Pregnancy	23	23	22	23	
Females					
Number in Group	24	24	24	24	
Number Not Pregnant	1	1	2	0	
Number Pregnant (%)	23 (95.8)	23 (95.8)	22 (91.8)	24 (100.0)	
Number Died/Killed before Pairing	0	0	0	0	
Number with Total Embryo/Foetal Loss	0	0	0	1	
Gestation Index (%)	100.0	100.0	100.0	95.8	F-
Number with Total Litter Loss	0	2	1	3	
Number with Live Pups at Day 21 post-partum	23	21	21	20	

F- = Cochram-Armitage and Fisher's Exact (lower tail)

Table A6_8_2-3: Summary of Adult Performance- F_1 Generation

Male/Female Performance	Group 1 Control	Group 2 250 ppm [redacted]	Group 3 500 ppm [redacted]	Group 4 1000 ppm [redacted]	Statistics
Males					
Number in Group	24	24	24	24	
Number Died/Killed before Pairing	0	0	0	0	
Number Paired	24	23 [a]	24	24	
Number Inducing Pregnancy	22	23	23	23	
Females					
Number in Group	24	24	24	24	
Number Died Before Pairing	0	1	0	0	
Number Not Pregnant	2	0	1	0	
Number Pregnant (%)	22 (91.7)	23 (100.0)	23 (95.8)	24 (100.0)	
Number Died/Killed before Pairing	0	0	0	0	
Number with Total Embryo/Foetal Loss	0	0	1	0	
Gestation Index (%)	100.0	100.0	95.8	100.0	F-
Number with Total Litter Loss	2	1	1	2	
Number with Live Pups at Day 21 post-partum	20	22	21	22	

[a] = Not paired due to death of allocated female
F- = Cochram-Armitage and Fisher's Exact (lower tail)

Table A6_8_2-4: Compound Consumption (P Generation)

Sex and Study Phase	[redacted] Dose Level (ppm)		
	250	500	1000
	Mean Intake of [redacted] (mg/kg/day)		
Males pre-pairing	18.5	37.2	75.1
Females pre-pairing	21.7	43.0	86.3
Females gestation	21.9	43.8	88.4
Females lactation #	37.5	75.8	160.9
Mean female	27.0	54.2	111.9
Sexes Combined	22.8	45.7	93.5

The mean value has been limited to Day 14 of lactation as subsequent compound consumption was influenced significantly by the offspring eating the test diets.

Table A6_8_2-5: Compound Consumption (F_1 Generation)

Sex and Study Phase	[redacted] Dose Level (ppm)		
	250	500	1000
	Mean Intake of [redacted] (mg/kg/day)		
Males pre-pairing	24.0	48.0	97.8
Females pre-pairing	27.4	55.6	115.7
Females gestation	20.9	41.4	84.0
Females lactation #	36.3	72.8	144.6
Mean female	28.2	56.6	114.8
Sexes Combined	26.1	52.3	106.3

The mean value has been limited to Day 14 of lactation as subsequent compound consumption was influenced significantly by the offspring eating the test diets.

Table A6_8_2-6: Table for Animal Assignment for Mating

Generation, m/f		[redacted] Dose Level (ppm)			
		Controls	Low Dose (250)	Medium Dose (500)	High Dose (1000)
		Number of Animals Paired for Mating			
Parents	m	24	24	24	23a
	f	24	24	24	24
F_1	m	24	23 b	24	24
	f	24	23a	24	24

[a] = Died before pairing
[b] = Not paired due to death of allocated female

Table A6_8_2-7: P Generation, F_1 Generation and F_2 Generation: Incidence of Mortality

Mortality (Incidence)	Generation	control		Low Dose 250 ppm		Medium Dose 500 ppm		High Dose 1000 ppm	
		m	f	m	f	m	f	m	f
	Parental	0	0	0	0	0	0	1a	0
	F_1	0	0	0	1b	0	0	0	0
	F_2	See Table A6_8_2-11							

[a] = 1 Male died before pairing (Animal number 73 was found dead at the beginning of Week 8 of the study. There was a large subcutaneous mass in the axillary region, swelling and blue colouration of the hind leg and general poor condition. Neeropsy examination showed that in addition to the mass, this animal had enlarged hind leg muscle, abnormal contents of the oesophagus, blood in the thoraeic cavity and markedly dark lungs.)
[b] = 1 Female died before pairing (Animal number 326 was killed in Week 9 of the pre-pairing period following clinical observation of teeth abnormalities and a swollen head. Necropsy examination confirmed the clinical observations but showed no other macroscopic abnormalities.)

Table A6_8_2-8: P Generation: Summary of Food Consumption, Body Weight, Organ Weights, Pathology and Histopathology Data

Parameter	Incidence	Control		Low Dose 250 ppm		Medium Dose 500 ppm		High Dose 1000 ppm	
		m	f	m	f	m	f	m	f
Food Consumption male week 1-10 (P)	% of control	100	NA	100.6	NA	101.3	NA	101.0	NA
Food Consumption female week 1-10 (P)	% of control	NA	100	NA	101	NA	100	NA	99.9
Food Consumption Gestation Day 0 -20 (P)	% of control	NA	100	NA	98.2	NA	98.7	NA	100
Food Consumption Lactation Day 1 -21 (P)	% of control	NA	100	NA	98.0	NA	98.8	NA	103
Body weight gain (P)a	% of control	100	100	98.7	98.4	98.8	98.0	96.2	98.3
Clinical Observations (P)	Incidence	No dose related observations recorded.							
Overall Organ weights (P)	% of control	100	100	101	97.0	98.6	97.8	102	95.5
Liver Weight (P)	% of control	100	100	105.9	100.5	100.1	104.8	108.2*	100.0
Testes (P)	% of control	100	NA	97.7	NA	96.5	NA	94.8**	NA
Pathology (P)	Stomach Raised Foci (incidence)	0/23	0/23	0/21	0/21	0/21	0/21	0/20	2/20
	Stomach Reddening (incidence)	0/23	0/23	0/21	1/21	0/21	2/21	0/20	2/20
	Stomach Thickening (incidence)	0/23	0/23	0/21	0/21	0/21	1/21	0/20	1/20
Histopathologic examination (P)	Limiting Ridge Hyperplasia (incidence)	0/24	0/24	0/24	0/24	5/24	8/24	16/23	16/24
	Squamous Cell Hyperplasia	0/24	0/24	0/24	0/24	0/24	1/24	0/23	3/24

	Forestomach Gastritis	0/24	0/24	0/24	0/24	0/24	0/24	1/23	1/24

NA = Not applicable
[a] = Body weight: Male and female body weight gain calculated from the mean group weights at the start of dosing to Week 18 and Day 21 of lactation, respectively.
- = Data not reported
* = Difference statistically significant when compared to the control group (P < 0.01, Dunnett's test). However, the female liver weight was unaffected by treatment and therefore the slight increase in male liver weight is not considered to represent an adverse effect of treatment.
** = Significant dose-related reduction in adjusted testes weight in high dose group (P < 0.05, Dunnett's test) However, in the absence of any effects on mating or seminology data, this is not considered to be an adverse effect of treatment.

Table A6_8_2-9: P Generation: Summary of Reproductive Performance Data

Parameter	Incidence	Control		Low Dose 250 ppm		Medium Dose 500 ppm		High Dose 1000 ppm	
		m	f	m	f	m	f	m	f
Mating index (%)	Mean	100		100		96.0		96.0	
Fertility index	Mean	95.8	95.8	95.8	95.8	91.7	91.7	100	100
Number of implantation sites	Mean	11.5		11.4		11.6		11.7	
Duration of pregnancy (days)	Mean	22.3		22.4		22.4		22.3	
Birth indexa	Mean	91.3		88.0		94.0		92.3	
Live birth index	Mean	98.3		100.0		98.0		98.9	
Gestation index	Mean	100.0		100.0		100.0		95.8	
Litter size	Mean	10.4		10.0		10.9		10.8	
Litter weight	Mean	-	-	-	-	-	-	-	-
Pup weight (g)b	Mean	5.5	5.1	5.5	5.3	5.6	5.2	5.4	5.0
Sex ratio	Male/female	1.07		0.815		1.01		0.637	
Survival index		-	-	-	-	-	-	-	-
Viability index (1%)		97.7		94.6		96.7		99.3	
Lactation index	Mean	-	-	-	-	-	-	-	-
Number	% of control	100		116.2		101.2		112.7	
Deformations (% not motile)	% of control	100		114		71.4		85.7	

Mean abnormal sperm (%)	% of control	100	-	-	13.3

[a] = Post implantation survival index
[b] = Mean weight day 1 (g)
- = Data not reported

Table A6_8_2-10: F_1 Generation: Summary of Food Consumption, Body Weight, Organ Weights, Pathology and Histopathology Data

Parameter	Incidence	Control		Low Dose 250 ppm		Medium Dose 500 ppm		High Dose 1000 ppm	
		m	f	m	f	m	f	m	f
Food Consumption male week 1-10 (F_1)	% of control	100	NA	100	NA	95.6	NA	98.5	NA
Food Consumption female week 1-10 (F_1)	% of control	NA	100	NA	97.5	NA	99.0	NA	103
Food Consumption Gestation Day 0 -20 (F_1)	% of control	NA	100	NA	99.5	NA	98.5	NA	99.5
Food Consumption Lactation Day 1 -21 (F_1)	% of control	NA	100	NA	101	NA	99.6	NA	98.9
Body weight gain (F_1)a	% of control	100	100	103	98.7	97.0	98.1	96.2	95.2
Clinical Observations (F_1)	Incidence	No dose related observations recoded.							
Organ weights (F_1)	% of control	100	100	96.1	101.8	94.3	101.5	96.6	99.7
Pathology (F_1)	Stomach Raised Foci (incidence)	0/24	0/24	0/24	0/24	0/24	0/24	0/24	2/22
	Raised Area (incidence)	0/24	0/24	0/24	0/24	0/24	0/24	1/24	0/24
	Stomach Reddening	0/24	4/20	0/24	2/22	0/24	4/21	0/24	6/22
	Stomach Thickening	0/24	0/24	0/24	0/24	0/24	0/24	0/24	22/24
Histopathologic examination (F_1)	Limiting Ridge Hyperplasia (incidence)	0/24	0/24	0/24	0/24	6/24	4/24	12/24	17/24
	Squamous Cell Hyperplasia	0/24	0/24	0/24	0/24	0/24	0/24	1/24	14/24
	Forestomach Gastritis	0/24	0/24	0/24	0/24	0/24	0/24	2/24	0/24

Parameter	Incidence	Control		Low Dose 250 ppm		Medium Dose 500 ppm		High Dose 1000 ppm	
		m	f	m	f	m	f	m	f
	Erosion/Ulcer	0/24	0/24	0/24	0/24	0/24	0/24	1/24	0/24
	Hyperkeratosis	0/24	0/24	0/24	0/24	0/24	0/24	7/24	0/24

NA = Not applicable
[a] = Body weight: Male and female body weight gain calculated from the mean group weights at the start of dosing to Week 18 and day 21 of lactation, respectively.
- = Data not reported

Table A6_8_2-11: F_1 Generation: Summary of Reproductive Performance Data

Parameter	Incidence	Control		Low Dose 250 ppm		Medium Dose 500 ppm		High Dose 1000 ppm	
		m	f	m	f	m	f	m	f
Mating index (%)		100		95.8		100.0		92.3	
Fertility index	Mean	91.7	91.7	100	100	95.8	95.8	95.8	100
Number of implantation sites c	Mean	11.3		10.9		10.7		10.2	
Duration of pregnancy (days)	Mean	22.2		22.3		22.2		22.2	
Birth index a	Mean	94.7		92.8		91.3		95.8	
Live birth index	Mean	97.1		94.7		98.7		97.7	
Gestation index	Mean	100.0		100.0		95.8		100.0	
Litter sizec	Mean	10.8		10.0		9.8		9.9	
Litter weight		-	-	-	-	-	-	-	-
Pup weight (g)b c	Mean	5.4	5.0	5.5	5.3	5.6	5.3	5.7	5.4
Sex ratio	Male/female	1.21		0.912		0.980		0.789	
Survival index		-	-	-	-	-	-	-	-
Viability index (1%)c	Mean	98.9		99.4		97.5		93.2	
Lactation index		-	-	-	-	-	-	-	-
Number (106/mL)	% of control	100		96.4		96.0		130.4	
Deformations (% not motile)	% of control	100		100		130		100	
Mean abnormal sperm (%)	% of control	100		-		-		41.7	

[a] = Post implantation survival index
[b] = Mean weight day 1 (g)
[c] = Significant Dose Response ($P < 0.05$)
- = Data not reported

Section A6	**Toxicological and Metabolic Studies**	
Subsection A6.9	**Neurotoxicity**	
Annex Point IIIA 6.9		
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ]	**Technically not feasible [ ]** **Scientifically unjustified [X]**	
Limited exposure [ ]	**Other justification [ ]**	
Detailed justification:	This is an additional data requirement, only required if there are any indications that the active substance may have neurotoxic properties. Indications of neurotoxicity can be acquired from the standard systemic toxicity studies. Acute toxicity studies with BIT showed no consistent pattern of behaviour indicative of a neurotoxic effect. Repeated dose studies (2-13 weeks) conducted with rodents and dogs via oral routes of exposure have reported no significant clinical observations whatsoever, with the exception of induced emesis and clinical chemistry and liver weight changes without any associated histopathological changes. None of the effects reported would implicate BIT as a nervous system toxicant. In addition, there are no structural alerts that would imply a potential for neurotoxic effects, nor any known metabolites that would cause neurotoxicity. Therefore, non-inclusion of this data is justified on the basis that the criteria for requirement are not met.	
Undertaking of intended data submission [ ]		

Evaluation by Competent Authorities

EVALUATION BY RAPPORTEUR MEMBER STATE

Date	*October 2008*
Evaluation of applicant's justification	*Applicant's justification is accepted.*
Conclusion	*Applicant is exempted of the neurotoxicity study.*
Remarks	*It is also remarkable, in addition to the applicant's justification, that the ADME studies did not show that BIT can cross the cerebral barrier and therefore effects of BIT on the neurons would be unlikely.*

Section A6	**Toxicological and Metabolic Studies**	
Subsection A6.10	**Mechanistic study**	
Annex Point IIIA 6.10		
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ]	**Technically not feasible [ ]** **Scientifically unjustified [X]**	
Limited exposure [ ]	**Other justification [ ]**	
Detailed justification:	This is an additional data requirement, and may be considered necessary when there are indications that active substance may have e.g. a non-genotoxic mechanism for carcinogenicity, species specific effects, adverse effects on reproduction, immunotoxicity or hormone related effects. Given the toxicological database for BIT that has been developed, there are no questionable toxicities or target organ effects that warrant additional mechanistic testing at this time. The use of additional animals for investigative testing is not supported or justified by hazard, exposure, or risk concerns. There are no acute, subchronic, or chronic hazard concerns or effects that have been identified for BIT that merit additional study for either elucidation of mechanism of action or establishment of no-effect levels for risk purposes. Therefore, non-inclusion of this data is justified on the basis that the criteria for requirement are not met.	
Undertaking of intended data submission [ ]		
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*October 2008*	
Evaluation of applicant's justification	*Applicant's justification is accepted.*	
Conclusion	*Applicant isexemted of the mechanistic studies.*	
Remarks		

Section A6	**Toxicological and Metabolic Studies**	
Subsection A6.11 **Annex Point IIIA 6.11**	**Studies on other routes of administration (parenteral routes)**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ] **Limited exposure [ ]**	**Technically not feasible [ ]** **Scientifically unjustified [X]** **Other justification [ ]**	
Detailed justification:	This is an additional data requirement, for existing substances, and data (if already existing) by alternative routes should be submitted. New studies will be required only in exceptional cases. No data on alternative routes exist for submission. Dermal exposure to BIT represents the most likely route of exposure based on use pattern. Accidental ingestion is possible. These routes of test material administration have been adequately investigated in current studies. Within the context of those studies that have been conducted, there are no clear toxicological concerns that would benefit from additional study using a parenteral route such as IV, SC, or IM. The metabolism study that has been conducted for BIT did not reveal unusual absorption, distribution, metabolic, or excretion data or profiles that warrant additional parenteral testing at this time. It is the scientific judgement of the Applicant that further parenteral testing will not yield data or information that would enhance our overall understanding of the toxicological characteristics of the active material. Therefore, non-inclusion of this data is justified on the basis that the criteria for requirement are not met.	
Undertaking of intended data submission [ ]		
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*October 2008*	
Evaluation of applicant's justification	*Applicant's justification is accepted.*	
Conclusion	*Applicant is exempted of studies on other routes of administration.*	
Remarks		

Section A6	**Toxicological and Metabolic Studies**	
Subsection A6.12.1 **Annex Point IIIA 6.12.1**	**Medical surveillance data on manufacturing plant personnel**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ]	**Technically not feasible [ ]** **Scientifically unjustified [X]**	
Limited exposure [ ]	**Other justification [ ]**	
Detailed justification:	If medical surveillance data are available, the Reports should be submitted. No reports are available for submission, therefore, non-inclusion of this data is justified on the basis that the criteria for requirement are not met. However, medical statements from a Corporate Medical Director (Arch Chemicals) and an Occupational Physician (Thor GmbH) are available, and have been submitted (IVA 6.12.1_1 and _2).	
Undertaking of intended data submission [ ]		
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*October 2008*	
Evaluation of applicant's justification	*Applicant's justification is accepted.*	
Conclusion	*Applicant is exempted to display medical surveillance data.*	
Remarks		

Section A6	**Toxicological and Metabolic Studies**	
Subsection A6.12.2	**Direct observation (clinical cases, poisoning incidents)**	
Annex Point IIIA 6.12.2		
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ]	**Technically not feasible [ ]** **Scientifically unjustified [X]**	
Limited exposure [ ]	**Other justification [ ]**	
Detailed justification:	If Direct observations, e.g. clinical cases, poisoning incidents, are available, the reports should be submitted. No reports are available for submission, therefore, non-inclusion of this data is justified on the basis that the criteria for requirement are not met.	
Undertaking of intended data submission [ ]		
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*October 2008*	
Evaluation of applicant's justification	*Applicant's justification is accepted.*	
Conclusion	*Applicant is exempted to display clinical cases or poisonings incidents.*	
Remarks		

Section A6	**Toxicological and Metabolic Studies**	
Subsection A6.12.3	**Medical data – Worker Health Incidences**	
Annex Point IIIA 6.12.3		
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ]	**Technically not feasible [ ]** **Scientifically unjustified [X]**	
Limited exposure [ ]	**Other justification [ ]**	
Detailed justification:	If Health records, both from industry and any other available sources, are available, the reports should be submitted. No reports are available for submission, therefore, non-inclusion of this data is justified on the basis that the criteria for requirement are not met. However, medical statements from a Corporate Medical Director (Arch Chemicals) and an Occupational Physician (Thor GmbH) are available, and have been submitted (IVA 6.12.1_1 and _2).	
Undertaking of intended data submission [ ]		
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*October 2008*	
Evaluation of applicant's justification	*Applicant's justification is accepted.*	
Conclusion	*Applicant is exempted to display health records.*	
Remarks		

Section A6 **Subsection A6.12.4** **Annex Point IIIA 6.12.4**	**Toxicological and Metabolic Studies** **EPIDEMIOLOGICAL STUDIES ON THE GENERAL POPULATION**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ] **Limited exposure [ ]**	**Technically not feasible [ ] Scientifically unjustified [X]** **Other justification [ ]**	
Detailed justification:	If Epidemiological studies on the general population, are available, the reports should be submitted. No reports are available for submission, therefore, non-inclusion of this data is justified on the basis that the criteria for requirement are not met.	
Undertaking of intended data submission [ ]		
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*October 2008*	
Evaluation of applicant's justification	*Applicant's justification is accepted.*	
Conclusion	*Applicant is exempted to display medical epidemiological studies.*	
Remarks		

Section A6	**Toxicological and Metabolic Studies**	
Subsection A6.12.5 **Annex Point IIAV1.6.9.5**	**DIAGNOSIS OF POISONING INCLUDING SPECIFIC SIGNS OF POISONING AND CLINICAL TESTS**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ] **Limited exposure [ ]**	**Technically not feasible [ ]** **Scientifically unjustified [ ]** **Other justification [X]**	
Detailed justification:	If information on diagnosis of poisoning including specific signs of poisoning and clinical test are available, the reports should be submitted. No reports are available for submission, therefore, non-inclusion of this data is justified on the basis that the criteria for requirement are not met.	
Undertaking of intended data submission [ ]		
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*Ocotber 2008.*	
Evaluation of applicant's justification	*Applicant's justification is accepted.*	
Conclusion	*Applicant is exempted to submit information on diagnosis of poisonings, specific signs of poisonings and clinical test.*	
Remarks		

Section A6 Toxicological and Metabolic Studies

Subsection A6.12.6(1) Human Case Report

Annex Point IIA VI.6.9.6 ***Occupational Asthma and Rhinitis observed after Exposure to 1,2-benzisothiazolin-3-one***

			Official use only
		1 REFERENCE	
1.1	**Reference**	Moscato G., Omodeo P., Dellabianca A., Colli C., Pugliese F., Locatelli C., Scibilia J.; 1997; Occupational Asthma and Rhinitis caused by 1,2-benzisothiazolin-3-one in a chemical worker. Occup. Med. Vol 47, 249-251, 1997.	
		2 GUIDELINES AND QUALITY ASSURANCE (NOT APPLICABLE)	
		3 MATERIALS AND METHODS	
3.1	**Substance**	Occupational Exposure: 1,2-benzisothiazolin-3-one (present in a microbicidal product). Challenge Test 1,2-benzisothiazolin-3-one (nebulized 0.04% aqueous solution).	
3.2	**Persons exposed**		
3.1.1	Sex	Male	
3.1.2	Age/weight	26 years (at the time of the challenge test). Weight not reported.	
3.1.3	Known Diseases	No personal or family history of allergic disease.	
3.1.4	Number of persons	1	
3.1.5	Other information		
3.3	**Exposure**	Inhalation	
3.3.1	Reason of exposure	Occupational and subsequent challenge test.	
3.3.2	Frequency of exposure	Occupational: Multiple. Three times per work shift for two weeks every month. Challenge: Single	

Section A6 Toxicological and Metabolic Studies

Subsection A6.12.6(1) Human Case Report

Annex Point IIA VI.6.9.6 ***Occupational Asthma and Rhinitis observed after Exposure to 1,2-benzisothiazolin-3-one***

3.3.3	Overall time period of exposure	Occupational: Exposure took place over the period of a year at the intervals detailed in 3.3.2.
3.3.4	Duration of single exposure	Challenge Test: 20 minutes
3.3.5	Exposure concentration/dose	Occupational: Data not available Challenge: 0.04 % aqueous solution of BIT; nebulised. The challenge was carried out in a static 7.2 cubic metre exposure chamber equipped for rapid air exchange. A fan in the chamber ensured adequate mixing and circulation.
3.3.6	Other information	Occupational Exposure involved exposure to BIT (present in a microbicidal product) in a chemical factory during production of detergents. The task consisted of pouring raw materials into the recipient of a mixing machine. The machine was equipped with a local exhaust fan on the feed chute. No data regarding the concentration of BIT in the biocidal product or the exposure levels were available.
3.4	**Examinations**	<u>Occupational</u> The patient was examined after 1 year of exposure (exposure described in 3.3.2 and 3.3.3). Examinations prior to the challenge test included: Physical examination; chest and sinus radiography; ECG; haematology; basal lung function tests, skin tests; tests for specific IgE levels for the common pneumoallergens and bronchial responsiveness.. <u>Challenge</u> General observations of symptoms. Spirometry was performed before and at 5, 15, 30 and 60 minutes after exposure. Thereafter at hourly intervals up to 7 hours after exposure. These lung function tests included (Peak Expiratory Flow (PEF) and Forced Expiratory Volume 1 (FEV1). The patient continued to take PEF measurements after 7 hours (throughout the evening, hourly or when symptoms occurred).
3.5	**Treatment**	The effect of treatment was not investigated however salbutamol (dosage or frequency of dose not stated) was administered during occupational exposure.

Section A6 Toxicological and Metabolic Studies

Subsection A6.12.6(1) Human Case Report

Annex Point IIA VI.6.9.6 ***Occupational Asthma and Rhinitis observed after Exposure to 1,2-benzisothiazolin-3-one***

3.6	**Remarks**	Occupational: The symptoms resolved promptly after inhalation of salbutamol.
		4 RESULTS
4.1	**Clinical Signs**	Occupational After 2 months exposure: Nasal itching and stuffiness, tearing, ocular burning and dry cough at the workplace. After 5 months exposure Sputum, dyspnoea and chest tightness in addition to the clinical signs above. Symptoms presented when the patient poured the raw materials into the machine; the symptoms spontaneously disappeared within 15 to 30 minutes. If the subject proceeded with the task then the symptoms persisted throughout the day, and sometimes appeared in the evening when away from work. Challenge Immediate prolonged asthmatic response Nasal symptoms (as detailed above during occupational exposure)
4.2	**Results of examinations**	Occupational Physical examination, chest radiograph and ECG were normal. Laboratory tests revealed mild blood eosinophilia (7%). The paranasal sinus radiograph showed bilateral mucoperiosteal thickening and deviation of the nasal septum. Basal lung function tests showed forced expiratory volume (FEV 1) of 107% predicted and vital capacity (VC) of 108% predicted. Both skin tests and the serum specific IgE levels for the more common pneumoallergens were negative. Total IgE were in the normal range. PD2O FEV1 of methacholine was greater than 3400 µg, indicating normal bronchial responsiveness, and ultrasonically nebulized distilled water challenge was negative. Challenge FEV l of 26% FEV 1 spontaneously returned to baseline values 2 h after the exposure and no late response was observed in the laboratory up to 7

hour later nor during the night.

4.3	**Effectivity of medical treatment**	Not applicable.
4.4	**Outcome**	Not applicable.
4.5	**Other**	Specific challenges were carried out with different substances (α-amylase, alcalase, polyacrylic acid or bezalkonium chloride) to which the patient was exposed at work (one challenge/day with a two day interval between each test). Each challenge was carried out in a static 7.2 cubic metre exposure chamber equipped for rapid air exchange. A fan in the chamber ensured adequate mixing and circulation. No significant variation in pulmonary function was observed when exposed to α-amylase, alcalase, polyacrylic acid or bezalkonium chloride.

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1	**Materials and methods**	A case of occupational asthma and rhinitis caused by inhalation of BIT an additive used as a biocide in detergent production was investigated in a 26-year-old man employed in a chemical factory producing detergents. The subject was exposed to BIT whilst pouring raw materials into the recipient of a mixing machine. Occupational exposure took place over the period of one year (three times per work shift for two weeks every month). Two months after the beginning of exposure the patient complained of rhinitis and asthma at the workplace. No data were available regarding exposure levels at the workplace A specific challenge test with BIT was performed one year after the occupational exposure commenced. Challenge tests with other agents (e.g., α-amylase, alcalase, polyacrylic acid or bezalkonium chloride) to which the subject was also exposed to during mixing and loading were performed.
5.2	**Results and discussion**	The specific challenge test with BIT, one of the raw materials to which the subject was exposed, provoked an immediate prolonged asthmatic response and nasal symptoms, whereas exposure to other agents (e.g., a-amylase, alcalase or bezalkonium chloride) to which the patient was also exposed at work did not.
5.3	**Conclusion**	This is a case of occupational asthma and rhinitis due to 1,2-benzisothia.zolin-3-one. As far as documented, this is the first case of occupational asthma and rhinitis caused by this compound.

Section A6 Toxicological and Metabolic Studies

Subsection A6.12.6(1) Human Case Report

Annex Point IIA VI.6.9.6 ***Occupational Asthma and Rhinitis observed after Exposure to 1,2-benzisothiazolin-3-one***

Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*October 2008.*
Materials and Methods	*Applicant's version is accepted.*
Results and discussion	*Applicant's version is accepted.*
Conclusion	*Applicant's conclusions are adopted.*
Remarks	*Minor mistake in section 4.1. The time of exposure was 3 months instead of 5 as is stated.*

Section A6 Toxicological and Metabolic Studies

Subsection A6.12.6(2) HUMAN CASE REPORT

Annex Point IIA VI.6.9.6 ***Human Repeat Insult Patch Test (HRIPT)***

Official use only

1 REFERENCE

1.1 Reference

[redacted]; 1975; Effect of 1,2 benzisothiazolin-3-one, 2-methyl-1,2 benzisothiazolin-3-one and Bis (2-methyl carbamoyl phenyl) disulphide on human skin. [redacted] and [redacted] Report No. [redacted]61/75763] [redacted]C/166

1.2 Data protection Yes

1.2.1 Data owner: Arch Chemicals Inc

1.2.2 Companies with letter of access: Clariant Production UK Ltd and Thor GmbH

1.2.3 Criteria for data protection: Data on existing substance for first entry into Annex I.

2 GUIDELINES AND QUALITY ASSURANCE (NOT APPLICABLE)

3 MATERIALS AND METHODS

Samples of three products, 1,2-Benzisothiazol-3-(2*H*)-one (BIT), 2-Methy-1, 2-Benzisothiazolin-3-one and Bis (2-Methyl Carbamoyl Phenyl) Disulphide were received in December 1974 for assessment of their irritancy and sensitisation potential to the skin of human volunteers.

This summary details the experimental procedure and results for the assessment of the sensitisation potential of BIT.

In a preliminary irritancy screen preceding a repeat insult patch test BIT at concentrations of 500, 750 and 1000 ppm was applied to the skin on three occasions in an attempt to identify a non-irritating dilution. The evaluation was made on ten, healthy, adult volunteers over a nine day period.

In this preliminary trial the concentration of BIT which could be applied without producing more than slight skin irritation was 500 ppm.

3.1 Substance 1,2-Benzisothiazol-3-(2*H*)-one (BIT).

Purity not stated.

3.2 Persons exposed

3.1.1 Sex: Male and Female

		(21 male and 29 female)
3.1.2	Age/weight	Age: subjects were aged 18 to 65 years Weight: Not reported
3.1.3	Known Diseases	Healthy volunteers Prior to commencing the test, each volunteer was asked a series of questions regarding any previous history of allergies. Only on the recommendation of the medical supervisor were they placed on the test.
3.1.4	Number of persons	Total of 50 volunteers
3.1.5	Other information	A group of 10 volunteers was treated as a pilot study prior to the main test with 40 volunteers. 5 volunteers failed to complete the study due to illness, hospitalisation or other engagements.
3.3	**Exposure**	Dermal
3.1.1	Reason of exposure	Volunteers in Human Repeat Insult Patch Test (HRIPT) Evaluation using a repeated insult patch test technique based on the method described by Shelanski, H.A. and Shelanski, M.V., Proceedings of the Scientific Section of Toilet Goods Association, 1953, vol. 19, pages 46-49.
3.1.2	Frequency of exposure	<u>Induction:</u> Patches were applied 3 times per week for 5 weeks. <u>First Challenge Test (2 weeks after induction)</u> One application. <u>Second Challenge Test (8 or 12 weeks after first challenge test)</u> One application (applied to volunteers showing evidence of possible sensitivity or atypical reactions to the first challenge).
3.1.3	Overall time period of exposure	<u>Induction</u> 24 hours per application. 15 applications over 3 weeks. Total of 15 days. <u>Challenge Tests</u> 24 hours
3.1.4	Duration of single exposure	24 hours

Section A6 Toxicological and Metabolic Studies

Subsection A6.12.6(2) HUMAN CASE REPORT

Annex Point IIA VI.6.9.6 ***Human Repeat Insult Patch Test (HRIPT)***

3.1.5	Exposure concentration/dose	0.5 mL of 500 ppm BIT solution applied to 1 of 4 patches (250 µg BIT/patch) Diameter of the patch = 7/8 inches (2.225 cm) Area of the patch = 3.879 cm^2 Exposure Concentration = 64.45 µg BIT/cm^2 0.5 mL of undiluted propylene glycol was applied to 1 of 4 patches. [2-Methy-1, 2-Benzisothiazolin-3-one and Bis (2-Methyl Carbamoyl Phenyl) Disulphide were applied to the other 2 patches. These compounds are not discussed in this summary]. <u>500 ppm BIT Solution</u> Prepared as follows: Prepared by weighing the appropriate amount of sample, which was then ground and added to the undiluted propylene glycol (with the exception of the second challenge test where BIT was suspended in liquid paraffin). This suspended solution was mixed on a Silverson stirrer machine but remained a suspension, 0.5 mL of BIT solution (500ppm) was added to each of 4 patches (Webril pads with 2.225 cm diameter) on a strip of Blenderm or Micropore surgical tape. (Micropore was used in 18 volunteers since reactions to the Blenderm tape were observed).
3.1.6	Other information	<u>Vehicle</u> The vehicles used are detailed below. A vehicle control was included in each test as appropriate. **Pilot Study** 100% propylene glycol was used in induction test and first and second challenge test. **Main Test** 100% propylene glycol was used in induction test and first challenge test. Liquid paraffin was used in second challenge test.
3.4	**Examinations**	<u>Induction</u> The sites were observed for reactions to treatment 24 or 48 hours after removal of the patches. <u>First and Second Challenge</u> The sites were examined 24 and 72 hours after removal of the patches. The reactions were graded according to the following scale:

0 - No reaction

I - Barely perceptible erytherna

2 - Faint but distinct erythema

3 - Slight erythema

4 - Moderate erythema

5 - Severe erythema

6 - Vesicular or weeping eczematous reactions

7 - Bullous reaction (spreading well outside the patch site)

E - Oedema, e.g. 2E - faint but distinct erythema with oedema

P - Papule formation

D - Dry flaking skin

3.5 Treatment Not applicable

3.6 Remarks

4 RESULTS

4.1 Clinical Signs

Induction (BIT in Propyleneglycol)

Propylene glycol and BIT elicited occasional dryness of the skin in most volunteers throughout the trial period

42 volunteers: Barely perceptible to slight erythema (associated with papule formation in six volunteers)

7 volunteers: Moderate erythema (accompanied by papule formation and/or oedema in three volunteers)

1 volunteer: Severe reaction (accompanied by papule formation) following the fourteenth application. Consequently, this volunteer did not receive the final induction application,

1 volunteer: Not re-patched following the thirteenth application since there were moderate to severe reactions observed at the other treated sites (these compounds are not discussed in this summary).

First challenge (BIT in Propyleneglycol)

45 of 50 volunteers received the challenge application to both arms (5 volunteers did not complete the study). The observations are summarised below:

9 volunteers: No dermal irritation was observed.

27 volunteers: Barely perceptible to slight erythema on the original and/or alternate arms following the challenge application. These reactions were essentially similar to those seen during the induction period and had generally ameliorated slightly at the 72 hour

observation.

*8 volunteers: Responses observed on the original arms, at the 24-hour observation (as strong, or stronger than occurred following the induction applications). Responses varied considerably from faint erythema to vesicular formation with oedema. Moderate erythema with or without papule formation and/or oedema occurred in 5 of these 8 volunteers.

*1 volunteer: Moderate erythema on the alternate arm and barely perceptible reaction only on the original arm at the 24 hour reading. - These reactions were considered atypical but had ameliorated by the 72 hour observation.

* Participated in the second challenge test

Weaker responses were generally observed on the alternate arms.

The reactions on both arms had generally ameliorated slightly by the 72 hour observation with the exception of one volunteer.

Second Challenge (BIT in Liquid Paraffin)

Performed to identify whether the reactions seen in the nine volunteers (*see previous page) in the first challenge application with 500 ppm BIT in propyleneglycol, were caused by irritation or sensitisation responses, a second challenge application was made eight or twelve weeks later.

The second challenge test was also performed on a further six volunteers who had atypical reactions to the other two test compounds (not discussed in this summary).

4 out of 9 volunteers: No dermal reactions occurred following the second challenge application.

5 out of 9 volunteers: Evidence of dermal sensitisation to BIT at 500 ppm as detailed below:

1 volunteer: Minimal dermal irritation was observed on both arms at the 24 hour observation and persisted until the 72 hour observation

3 volunteers: Marked reactions indicative of dermal sensitisation including vesicular reactions, with or without oedema, on the original or both arms during the 72-hour observation period. One of these volunteers showed moderate erythema accompanied by papule formation and oedema on the original arm at the 24 hour reading and barely perceptible erythema with papule formation on the alternate arm.

1 volunteer: slight erythema associated with papule formation which persisted throughout the 72 hour challenge period.

No dermal Irritation was seen on the control patch sites treated with liquid paraffin.

Section A6 Toxicological and Metabolic Studies

Subsection A6.12.6(2) HUMAN CASE REPORT

Annex Point IIA VI.6.9.6 ***Human Repeat Insult Patch Test (HRIPT)***

4.2	**Results of examinations**	Not Applicable
4.3	**Effectivity of medical treatment**	Not Applicable
4.4	**Outcome**	Not Applicable
4.5	**Other**	Not Applicable

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and methods

A human skin sensitisation study was performed using the Human Repeat Insult Patch Test (HRIPT) methodology. The study was conducted on three biocides, including BIT. BIT was applied to 45 volunteers using an induction regime of 15 applications over a 5 week period (three 24 hr induction dose per week) at a concentration of 500 ppm in propylene glycol. The exposure concentration was 64.45 µg BIT/cm^2.

Two weeks after the induction period, a challenge application of 500 ppm BIT was applied to each volunteer. Where necessary, a second challenge application was also made, eight to twelve weeks later, to volunteers showing evidence of possible sensitivity or atypical reactions in response to the first challenge.

5.2 Results and discussion

BIT had the potential to cause irritation reactions during the induction period in the majority of the volunteers. The degree of severity was mainly of a mild nature (seven volunteers) but moderate or severe irritation was seen in one volunteer.

At challenge, mild irritation was seen in 27/45 volunteers, of a similar degree to that seen during the induction phase. In nine volunteers, the reaction was as great or greater than that seen previously. However, the vehicle propyleneglycol, also elicited dermal irritation which was greater than expected.

In order to clarify the results obtained from the first challenge, a second challenge application was made to these nine volunteers, and in addition, six other volunteers also received the second challenge application because of atypical reactions produced by the other test compounds following the first challenge. Therefore fifteen volunteers were rechallenged using liquid paraffin as the vehicle. Marked reactions indicative of dermal sensitisation were observed in five individuals following the second challenge.

BIT produced evidence of dermal sensitisation in five volunteers. There was also evidence of slight dermal irritation in most individuals. Therefore, BIT has the potential to cause skin sensitisation in humans.

Section A6 Toxicological and Metabolic Studies

Subsection A6.12.6(2) HUMAN CASE REPORT

Annex Point IIA VI.6.9.6 ***Human Repeat Insult Patch Test (HRIPT)***

5.3	**Conclusion**	From the study data it can be concluded that BIT at an exposure concentration was 64.45 µg BIT/cm^2 has the potential to cause skin sensitisation in humans. Since the sensitisation study with BIT was carried out, propylene glycol has been demonstrated to be a penetration enhancer. It is believed that the effect of formulating BIT in propylene glycol facilitated absorption and accentuated the sensitisation response. However, the study did confirm the intrinsic capacity of BIT to cause human sensitisation.

Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*October 2008.*
Materials and Methods	*Applicant's version is accepted.*
Results and discussion	*Applicant's version is accepted.*
Conclusion	*Applicant's conclusions are adopted.*
Remarks	**--**

Section A6 Toxicological and Metabolic Studies

Subsection A6.12.6(3) HUMAN CASE REPORT

Annex Point IIA VI.6.9.6 ***Human Repeat Insult Patch Test (HRIPT)***

Official use only

1 REFERENCE

1.1 Reference [redacted] 1972; Repeat Insult Patch Test of [redacted] (360 ppm). [redacted] Report No. [72-462] Report No. [redacted]C/2993; Not GLP; Unpublished.

1.2 Data protection Yes

1.2.1 Data owner: Arch Chemicals Inc

1.2.2 Companies with letter of access: Clariant Production UK Ltd and Thor GmbH

1.2.3 Criteria for data protection: Data on existing substance for first entry into Annex I.

2 GUIDELINES AND QUALITY ASSURANCE (NOT APPLICABLE)

3 MATERIALS AND METHODS

3.1 Substance [redacted] (33 % BIT)

3.2 Persons exposed

3.1.1 Sex: Male and Female

(5 male and 49 female)

3.1.2 Age/weight: Age: as detailed below

Sex	16-20	21-30	31-40	41-50	> 60
	Years				
Female	2	11	25	10	1
Male	0	1	1	2	1

Weight: Not reported

3.1.3 Known Diseases: None reported

3.1.4 Number of persons: Total of 56 subjects

3.1.5 Other information: 54 subjects completed the study (one subject did not complete the study due to excessive absence and another subject dropped out due to

		illness).
3.3	**Exposure**	Dermal
3.1.1	Reason of exposure	Volunteers in Human Repeat Insult Patch Test (HRIPT) The procedure used was an adaptation of J. H. Draize, "Dermal Toxicity," in Appraisa1 of the Safety of Chemicals in Foods, Drugs and Cosmetics, The Staff of the Division of Pharmacology of the Federal Food and Drug Administration (Austin,Texas: The Editorial Committee of the Association of Food and Drug Officials of the United States; 1959).
3.1.2	Frequency of exposure	Serial Application 3 times per week for 3 weeks. Challenge Test One challenge test was performed. (2 weeks after last of the serial applications duplicate patches were applied. One patch was applied to the original site and the other to a site not previously exposed).
3.1.3	Overall time period of exposure	Serial Application 24 hours per application. 9 applications over 3 weeks. Total of 9 days. Challenge Test 24 hours
3.1.4	Duration of single exposure	24 hours
3.1.5	Exposure concentration/dose	0.5 mL of 360 ppm BIT in water was applied to swatch of Webril.* (180 µg BIT/patch) Diameter of the patch = 3/4 × 7/8 inches (1.905 × 2.225 cm) Area of the patch = 4.239 cm^2 Exposure Concentration = 42.47 µg BIT/cm^2 *The test patch consisted of a 3/4 x 7/8 inch swatch of Webril (absorbent non-woven cotton fabric) affixed to the centre of an elastic adhesive bandage. The patches were specially prepared for the test.
3.1.6	Other information	Vehicle: Water The test material was diluted with water to 1087 ppm [redacted] which is equivalent to 360 ppm BIT considering an active ingredient content of 33%.* Fresh solutions were made, shortly before each application.

A patch of the test material was applied to the right upper arm of each subject three times per week for three consecutive weeks.

The subjects were instructed to remove the patches 24 hours after application. Applications were made to the same site a severe reaction was observed. In such cases applications were made to fresh adjacent sites.

Duplicate challenge applications of the test material were made two weeks after the final serial application, one set of patches to the original sites and one to adjacent sites which had not been previously patched.

* Hill Top Research Report No. 72-462 stares that the BIT concentration of the 1087 ppm [redacted] solution is 250 ppm. This concentration was corrected to 360 ppm when the report was issued as [redacted]C/2993. Information from the supplier of [redacted] confirmed that the test item contained 33% BIT and not 23% BIT as stated in [redacted] Report No. 72-462.

3.4 Examinations

Serial Application

The patch sites were scored by an experienced staff member just prior to the patch applications from the second to tenth visit (inclusive).

Challenge Test

The challenge application sites were scored at 48 and 96 hours after application.

Scoring

All readings were made under light supplied by a 100-watt incandescent blue bulb, Scoring was performed according to the following scale:

0: No evidence of irritation

1: Slight erythema

2: Marked erythena

3: Erythema and papules

4: Edema; erythema may also be present

5: Erythema, edema, and papules

6: Vesicular eruption

7: Strong reaction spreading beyond test site

Surface Effects

A: Slight glazed appearance

B: Marked glazing

C: Glazing with peeling and cracking

		F: Glazing with fissures G: Film of dried serous exudate covering all or portions of the patch site H: Small petechial erosions and/or scabs
3.5	**Treatment**	Not applicable
3.6	**Remarks**	None
		4 RESULTS
4.1	**Clinical Signs**	No irritation was observed at any time during the study.
4.2	**Results of examinations**	Not Applicable
4.3	**Effectivity of medical treatment**	Not Applicable
4.4	**Outcome**	Not Applicable
4.5	**Other**	Not Applicable
		5 APPLICANT'S SUMMARY AND CONCLUSION
5.1	**Materials and methods**	The skin sensitisation and skin irritation potential of [redacted] a formulation containing 33% BIT (and 20.5-22.5% ethylene diamine), was assessed using the Human Repeat Insult Patch Test. In the initial study, 54 volunteers each received ten applications of a solution of [redacted] containing 1087 ppm of the test material (equivalent to 360 ppm BIT) in water. The exposure concentration was 42.47 µg BIT/cm^2. The patch sites were scored by an experienced staff member just prior to the patch applications from the second to tenth visit (inclusive). Duplicate challenge applications of the same solution were made two weeks after the final serial applications, one set of patches to the original sites and one to adjacent sites which had not been previously patched. The challenge application sites were scored at 48 and 96 hours after application.
5.2	**Results and discussion**	The test solution was found to be non-irritant and not to be a contact sensitiser.
5.3	**Conclusion**	BIT at an exposure concentration of 42.47 µg BIT/cm^2 was found not induce sensitisation.

Section A6 **Toxicological and Metabolic Studies**

Subsection A6.12.6(3) **HUMAN CASE REPORT**

Annex Point IIA VI.6.9.6 ***Human Repeat Insult Patch Test (HRIPT)***

Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*October 2008.*
Materials and Methods	*Applicant's version is accepted.*
Results and discussion	*Applicant's version is accepted.*
Conclusion	*Applicant's conclusions are adopted.*
Remarks	--

Section A6	**Toxicological and Metabolic Studies**	
Subsection A6.12.7 **Annex Point IIAV1.6.9.7**	**SPECIFIC TREATMENT IN CASE OF AN ACCIDENT OR POISONING: FIRST AID MEASURE, ANTIDOTES AND MEDICAL TREATMENT, IF KNOWN**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ] **Limited exposure [ ]**	**Technically not feasible [ ]** **Scientifically unjustified [ ]** **Other justification [X]**	
Detailed justification:	Inhalation: Remove patient from exposure, keep warm and at rest. Obtain medical attention if ill effects occur. Skin contact: Take off immediately all contaminated clothing. Wash immediately with tepid or cold water followed by soap and water. Obtain medical attention. Contaminated clothing should be laundered before re-issue. Eye contact: SPEED IS ESSENTIAL. OBTAIN IMMEDIATE MEDICAL ATTENTION. Immediately irrigate with eyewash solution or clean water, holding the eyelids apart, for at least 15 minutes. Continue irrigation until medical attention can be obtained. Ingestion: Provided the patient is conscious, wash out mouth with water and give 200-300 mL (half a pint) of water to drink. Do not induce vomiting. Obtain medical attention. Notes to physician Treatment: Symptomatic treatment and supportive therapy as indicated.	
Undertaking of intended data submission [ ]		

Evaluation by Competent Authorities

EVALUATION BY RAPPORTEUR MEMBER STATE

Date	*October 2008.*
Evaluation of applicant's justification	*Applicant's justification is accepted.*
Conclusion	*The proposed treatments seems to be appropriate to deal with poisonings of irritants as BIT.*
Remarks	

Section A6	**Toxicological and Metabolic Studies**	
Subsection A6.12.8	**PROGNOSIS FOLLOWING POISONING**	
Annex Point IIA6.9.8		
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ]	**Technically not feasible [ ]** **Scientifically unjustified [ ]**	
Limited exposure [ ]	**Other justification [X]**	
Detailed justification:	There are no known cases of poisoning with BIT.	
Undertaking of intended data submission [ ]		
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*October 2008*	
Evaluation of applicant's justification	*Applicant's justification is accepted.*	
Conclusion	*Since there are no cases of poisonings with BIT applicants cannot supply information about prognosis.*	
Remarks		

Section A6	**Toxicological and Metabolic Studies**	
Subsection A6.13	**TOXIC EFFECTS ON LIVESTOCK AND PETS**	
Annex Point IIIA VI. 2		
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [X]	**Technically not feasible [ ]** **Scientifically unjustified [ ]**	
Limited exposure [X]	**Other justification [ ]**	
Detailed justification:	In relevant, but exceptional cases, toxicity testing in livestock and pets is required. This is an additional data requirement, which is recognised by the TNsG on data requirements as possibly relevant for several product types, but is usually not required for the product types 1, 2, 6, 7, 9, 11, 12, 13, 20, 21 and 22. The uses being supported for BIT indicate little relevancy for this testing, therefore non-inclusion of this data is justified on the basis that the criteria for requirement are not met.	
Undertaking of intended data submission [ ]		
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*November 2008*	
Evaluation of applicant's justification	*Applicant's justification is accepted.*	
Conclusion	*Applicant is exempted to perform assays of toxic effects on livestock and pets.*	
Remarks		

Section A6	**Toxicological and Metabolic Studies**	
Subsection A6.14	**OTHER TESTS RELATED TO THE EXPOSURE OF HUMANS**	
Annex Point IIIA 6.14		
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ]	**Technically not feasible [ ] Scientifically unjustified [X]**	
Limited exposure [ ]	**Other justification [ ]**	
Detailed justification:	The potential for human exposure to substances generated from the active substance other than mammalian metabolites is not considered to be significant in normal use. Consequently it is not necessary to assess the toxicity of such substances. Therefore non-inclusion of this data is justified on the basis that the criteria for requirement are not met.	
Undertaking of intended data submission [ ]		
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*November 2008*	
Evaluation of applicant's justification	*Applicant's justification is accepted.*	
Conclusion	*Applicant is exempted to perform other test related to the exposure of humans.*	
Remarks		

Section A6	**Toxicological and Metabolic Studies**	
Subsection A6.15/1 **Annex Point IIIA XI.1.1, 1.3, 1.6**	**IDENTIFICATION OF THE RESIDUES (IDENTITY AND CONCENTRATIONS), DEGRADATION AND REACTION PRODUCTS AND OF METABOLITES OF THE ACTIVE SUBSTANCE IN CONTAMINATED FOODS OR FEEDING STUFFS**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ] **Limited exposure [ ]**	**Technically not feasible [ ] Scientifically unjustified [ ]** **Other justification [X]**	
Detailed justification:	The test substance will not come into contact with food and feedstuffs. No further tests on residues, degradation and reaction products and metabolites of the active substance in contaminated foods or feeding stuffs are therefore considered necessary. Therefore, non-inclusion of this data is justified on the basis that the criteria for requirement are not met.	
Undertaking of intended data submission [ ]		
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*November 2008*	
Evaluation of applicant's justification	*Applicant's justification is accepted.*	
Conclusion	*Applicant is exempted to perform the identification of the residues, degradation and reaction products and of metabolites of the active substance in contaminated food and feedingstuffs.*	
Remarks		

Section A6	**Toxicological and Metabolic Studies**	
Subsection A6.15/2 **Annex Point IIIA XI.1.2, 1.3, 1.5, 1.6**	**BEHAVIOUR OF THE RESIDUES OF THE ACTIVE SUBSTANCE, ITS DEGRADATION AND REACTION PRODUCTS AND WHERE RELEVANT, ITS METABOLITES ON THE TREATED OR CONTAMINATED FOOD OR FEEDING STUFFS INCLUDING THE KINETICS OF DISAPPEARANCE**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ] **Limited exposure [ ]**	**Technically not feasible [ ] Scientifically unjustified [ ]** **Other justification [X]**	
Detailed justification:	The test substance will not come into contact with food and feedstuffs. No further tests on residues, degradation and reaction products and metabolites of the active substance in contaminated foods or feeding stuffs are therefore considered necessary. Therefore, non-inclusion of this data is justified on the basis that the criteria for requirement are not met.	
Undertaking of intended data submission [ ]		
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*November 2008*	
Evaluation of applicant's justification	*Applicant justification is accepted.*	
Conclusion	*Applicant is exempted to study behaviour of the residues of the active substance, its degradation and reaction products and where relevant, its metabolites on the treated or contaminated food and feedingstuffs.*	
Remarks		

Section A6	**Toxicological and Metabolic Studies**	
Subsection A6.15/3 **Annex Point IIIA XI.1.4**	**ESTIMATION OF POTENTIAL OR ACTUAL EXPOSURE OF THE ACTIVE SUBSTANCE TO HUMANS THROUGH DIET AND OTHER MEANS**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ] **Limited exposure [ ]**	**Technically not feasible [ ]** **Scientifically unjustified [ ]** **Other justification [X]**	
Detailed justification:	The test substance will not come into contact with food and feedstuffs. Estimations of potential or actual exposure of the active substance to humans through diet and other means are therefore considered unnecessary. Therefore, non-inclusion of this data is justified on the basis that the criteria for requirement are not met.	
Undertaking of intended data submission [ ]		
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*November 2008*	
Evaluation of applicant's justification	*Applicant's justification is accepted.*	
Conclusion	*Applicant is exempted to estimate the potential or actual exposure of the active substance of humans through diet and other means.*	
Remarks		

Section A6	**Toxicological and Metabolic Studies**	
Subsection A6.15/4 **Annex Point IIIA XI.1.7**	**PROPOSED ACCEPTABLE RESIDUES AND THE JUSTIFICATION OF THEIR ACCEPTABILITY**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ] **Limited exposure [ ]**	**Technically not feasible [ ] Scientifically unjustified [ ]** **Other justification [X]**	
Detailed justification:	The test substance will not come into contact with food and feedstuffs. Proposed acceptable residues and the justification of their acceptability are therefore considered unnecessary. Therefore, non-inclusion of this data is justified on the basis that the criteria for requirement are not met.	
Undertaking of intended data submission [ ]		
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*November 2008*	
Evaluation of applicant's justification	*Applicant's justification is accepted.*	
Conclusion	*Applicant is exempted to supply ……..*	
Remarks		

Section A6 **Subsection A6.15/5** **Annex Point IIIA XI.1.8**	**Toxicological and Metabolic Studies** **ANY OTHER AVAILABLE INFORMATION THAT IS RELEVANT**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ] **Limited exposure [ ]**	**Technically not feasible [ ] Scientifically unjustified [ ]** **Other justification [X]**	
Detailed justification:	The test substance will not come into contact with food and feedstuffs. No other information is therefore provided. Therefore, non-inclusion of this data is justified on the basis that the criteria for requirement are not met.	
Undertaking of intended data submission [ ]		
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*November 2008*	
Evaluation of applicant's justification	*Applicant's justification is accepted.*	
Conclusion	*Applicant is exempted to supply any other information.*	
Remarks		

Section A6	**Toxicological and Metabolic Studies**	
Subsection A6.15/6 **Annex Point IIIA XI.1.9**	**SUMMARY AND EVALUATION OF DATA SUBMITTED UNDER POINT 6.15**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ] **Limited exposure [ ]**	**Technically not feasible [ ] Scientifically unjustified [ ]** **Other justification [X]**	
Detailed justification:	The test substance should not come into contact with food and feeding stuffs. As no data have been produced or evaluated, no summary or evaluation are therefore provided in Doc. III-A, TNG Section A6.15.	
Undertaking of intended data submission [ ]		
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*November 2008*	
Evaluation of applicant's justification	*Applicant`s justification is accepted.*	
Conclusion	*Applicant is exempted to supply a summary and evaluation of data submitted under point 6.15.*	
Remarks		

Section A6	**Toxicological and Metabolic Studies**	
Subsection A6.16 **Annex Point IIIA6.3.5-IIIA11.2**	**ANY OTHER TESTS RELATED TO THE EXPOSURE OF THE ACTIVE SUBSTANCE TO HUMANS, IN ITS PROPOSED BIOCIDAL PRODUCTS, THAT ARE CONSIDERED NECESSARY**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ] **Limited exposure [ ]**	**Technically not feasible [ ]** **Scientifically unjustified [ ]** **Other justification [X]**	
Detailed justification:	Based on an evaluation of the available information on the active substance, it is considered that no further tests related to the exposure of the active substance to humans are necessary. Risk assessments have been performed for each usage and are contained in Doc. II-B.	
Undertaking of intended data submission [ ]		
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*November 2008*	
Evaluation of applicant's justification	*Applicant's justification is accepted.*	
Conclusion	*Applicant is exempted to perform any other test related to the exposure of the active substance to humans.*	
Remarks		

Section A6 **Subsection A6.17** **Annex Point IIIA6.17**	**Toxicological and Metabolic Studies** **IF ACTIVE SUBSTANCE IS TO BE USED IN PRODUCTS FOR ACTION AGAINST PLANTS THEN TESTS TO ASSESS TOXIC EFFECTS OF METABOLITES FROM TREATED PLANTS.**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ] **Limited exposure [ ]**	**Technically not feasible [ ] Scientifically unjustified [ ]** **Other justification [X]**	
Detailed justification:	BIT is not sold for use against plants. Therefore, non-inclusion of this data is justified on the basis that the criteria for requirement are not met.	
Undertaking of intended data submission [ ]		
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*November 2008*	
Evaluation of applicant's justification	*Applicant's justification is accepted.*	
Conclusion	*Applicant is exempted to assess toxic effects of metabolites from treated plants.*	
Remarks		

Section A6	**Toxicological and Metabolic Studies**	
Subsection A6.18 **Annex Point IIIAVI.6**	**SUMMARY OF MAMMALIAN TOXICOLOGY AND CONCLUSIONS**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ] **Limited exposure [ ]**	**Technically not feasible [ ]** **Scientifically unjustified [X]** **Other justification [ ]**	
Detailed justification:	**Toxicokinetics** In studies with rats, 1,2-Benzisothiazol-3-(2*H*)-one was rapidly and extensively absorbed through the skin and from the gastrointestinal tract. At 8 hours after an oral administration, 96.6% of the radiochemical dose was detected in samples other than the gastrointestinal tract. At 8 hours after a topical application (most representative timepoint for exposure during a working day), 3.2% of the radiochemical dose was absorbed and 23.9% remained in the treated skin and was, therefore, available for absorption. At 72 hours after a topical application, 41.6% of the radiochemical dose was absorbed and 47.6% remained in the treated skin and was, therefore, available for absorption. The primary route of excretion was in the urine, with 96.6% of the activity absorbed following topical application and 99.5% of the radiochemical dose following oral administration, being excreted by this route in 72 hours. Very little material was detected in the faeces, indicating that the majority of the radioactive material is absorbed following oral administration and that biliary excretion is unlikely to occur to any great extent (less than 0.5% of the radiochemical dose combined were detected in the faeces and gastrointestinal tract following topical application). The test material does not appear to be broken down to volatile components or excreted in the expired carbon dioxide, as indicated by high overall recoveries and low trap levels of radioactivity in trapping solutions (less than 0.05% of the radiochemical dose). Tissue disposition does not appear to occur. Less than 0.05% of the radiochemical dose remained in any tissue at 48 hours after oral administration and topical application, with the exception of the carcass and untreated skin following topical application which, combined, contained less than 1.5% of the radiochemical dose. The metabolic routes in dogs and rats are essentially similar. The breakdown of BIT by both species is rapid and is carried virtually to completion, since no unchanged BIT was found in either dog or rat urine. The major urinary metabolites of both species appear to result from the reduction of the nitrogen-sulphur bond, followed by methylation and oxidation of the sulphur atom. Three metabolites were present, although it was not possible to identifiy metabolite 1. Metabolite 2 was identified as *o*-(methylsulphinyl) benzamide, and	

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metabolite 3 as *o*-(methylsulphonyl) benzamide.

Acute toxicity

1,2-Benzisothiazol-3-(2*H*)-one (BIT) is of moderate acute toxicity via the oral route. The acute oral lethal dose (LD_{50}) in rats is calculated to be 670 and 784 mg/kg for males and females (equivalent to 490 and 573 mg/kg BIT, respectively). The LD_{50} in male rats in another oral gavage study was in close agreement since an LD_{50} of 454 mg/kg BIT was obtained. In this study female rats were dosed at the lowest dose rate (202 mg/kg) and in common with the male animals there was no sign of reaction to treatment at this dose rate. Corresponding EU classification is as 'Harmful', with assignation of the hazard symbol 'Xn' and risk phrase 'R22 Harmful if swallowed', in conformity with BIT's classification in Annex I to the Dangerous Substances Directive 67/54/EEC as amended.

As neither the pure nor the technical grade active substance is a gas or volatile liquid, and as the nature of the technical grade active substance (a paste) makes the most likely route of exposure dermal, in accordance with the guidance at Technical Guidance on data requirements, Ch. 2: Core data set / Part A, 6.1 Acute Toxicity [Ann IIA, VI. 6.1.], the most appropriate second route of administration is dermal.

In a dermal toxicity study where rats were exposed to Technical Grade BIT at 2000mg/kg there was no reaction to treatment observed. The acute dermal LD_{50} is therefore > 2000 mg/kg Technical Grade BIT (equivalent to > 1462 mg/kg BIT). The TGAS is not EU classifiable for acute dermal toxicity on this basis.

Irritation and Corrositivity

Technical Grade BIT was slightly irritant to rabbit skin following a four-hour exposure; it is not EU classifiable for skin irritation on this basis.

BIT's classification in Annex I to the Dangerous Substances Directive 67/54/EEC as amended is as 'Irritant', with assignation of the hazard symbol 'Xi' and risk phrase 'R38 Irritating to skin'.

Technical Grade BIT was a very severe irritant to the rabbit eye and should be regarded as at least an extremely severe ocular irritant. Corresponding EU classification is as 'Irritant', with assignation of the hazard symbol 'Xi' and risk phrase 'R41 Risk of serious damage to eyes', in conformity with BIT's classification in Annex I to the Dangerous Substances Directive 67/54/EEC as amended.

Skin Sensitisation

Challenge of previously induced guinea pigs with a 10% (w/v) preparation of BIT elicited a moderate skin sensitisation response and challenge with a 3% (w/v) preparation elicited a mild skin sensitisation response. BIT was a moderate skin sensitiser under the conditions of the test. Corresponding EU classification is as 'Irritant',

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with assignation of the hazard symbol 'Xi' and risk phrase 'R43 May cause sensitization by skin contact', in conformity with BIT's classification in Annex I to the Dangerous Substances Directive 67/54/EEC as amended.

Repeat dose toxicity

In a 28 day oral gavage study in rats Technical Grade BIT at dosages of 50 or 150 mg/kg/day produced changes in the stomach that were consistent with a response to an irritant material and all other findings on this study could be attributed to this response. The no-observed-effect level (NO(A)EL) in this study was 15 mg/kg/day.

In a 90 day rat feeding study with dose groups of 200, 900 and 4000 ppm groups (mean dose rates of 15.3, 69.0 and 322 mg/kg/day in males and 17.6, 78.3 and 356 mg/kg/day in females, respectively) the lowest concentration with observed effects (LO(A)EL) was 4000 ppm (equivalent to 322.0 and 356.3 mg/kg/day in males and females, respectively). The effects at this dose level included impaired growth rate and histopathological changes in the stomach.

In the equivalent study in dogs with dose groups of 5, 20 and 50 mg/kg/day, no effects were observed at 5 mg/kg/day. The LO(A)EL was 20 mg/kg/day (characterised by emesis, clinical chemistry and liver weight changes without any associated histopathological changes). The NO(A)EL was 5 mg/kg.

Genotoxicity

Technical Grade BIT has been examined in a number of short term assays for genotoxicity. In the Bacterial Mutation assay, isolated increases in revertants were observed in some Salmonella strains, although the lack of reproducibility and dose response usually seen suggests that this is not a true chemically mediated effect. This is supported by the negative response seen in the L5178Y mammalian cell gene mutation assay.

Technical Grade BIT produced a negative response in the in vivo mouse bone marrow micronucleus assay and the rat-liver UDS assay, indicating that the compound is not metabolised to a mutagenic species in vivo.

The overall conclusion from these assays, together with the chemical structure of the active component which does not contain any features alerting to possible genotoxicity, is that Technical Grade BIT presents no genotoxic hazard.

Combined Chronic toxicity and Carcinogenicity

The lack of mutagenicity in vivo for BIT, the lack of carcinogenicity of other isothiazolinone derivatives, the similar toxicological profile observed for isothiazolinones following chronic exposure, and the prediction that BIT would not be carcinogenic based on SAR analyses, together provide significant weight of evidence support for the conclusion that this chemical is predicted not to be associated with

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carcinogenic potential and that a chronic toxicity test and a cancer bioassay are unnecessary to characterise its intrinsic health hazard.

Reproductive toxicity

In a rat teratology study, Technical Grade BIT did not cause any developmentally toxic effects at a maternally toxic dose level. Marginal foetotoxicity was observed at the highest dose level tested (100 mg/kg/day), in the presence of maternal toxicity. The NO(A)EL for maternal toxicity was 10 mg/kg/day and for foetotoxicity 40 mg/kg/day.

BIT showed no teratogenic effects in rats. In addition, BIT caused no developmental toxic effects in a two-generation reproduction toxicity study in rats. Structurally similar isothiazolinones caused no teratogenic effects in two teratogenicity studies in rats and three teratogenicity studies in rabbits. It can be concluded that the isothiazolinones as a class lack the potential to cause developmental toxicity and, thus, that sufficient information is available to complete a risk assessment of BIT without generating teratogenicity data in a second species (rabbit). This conclusion is supported by the qualitatively and quantitatively similar toxicological profile for BIT and other isothiazolinones, including the observations that they produce minor effects upon repeated exposure and lack genotoxic potential.

In a two-generation reproduction study in the rat the parental LO(A)EL was 500 ppm (mean dose of 37.2 and 54.2 mg/kg/day in males and females, respectively) based on toxic effects on the stomach (hyperplasia of the limiting ridge of the stomach). The LO(A)EL for the F_1 generation was 1000 ppm (mean dose of 97.8 mg/kg/day and 114.8 mg/kg/day in males and females, respectively) based on impaired growth and survival of pups.

Neurotoxicity

In the 28 day rat oral toxicity study, no treatment-related changes were identified in the sensory reactivity tests or grip strength and motor activity measurements performed in Week 4.

Human Data

Occupational asthma and rhinitis attributed to inhalation of BIT was observed in a 26 year old male. In a specific challenge perfomed one year after occupational exposure BIT provoked an immediate prolonged asthmatic response and nasal symptoms. As far as is documented, this is the first and only case of occupational asthma and rhinitis attributed to inhalation of BIT.

In a skin sensitisation study performed using the Human Repeat Insult Patch Test (HRIPT) methodology there was no evidence of sensitisation at an exposure rate of 42.47 µg BIT/cm^2. In another HRIPT study it was concluded that BIT at an exposure rate of 64.45 µg BIT/cm^2 has the potential to cause skin sensitisation in humans.

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	In common with other isothiazolinones, BIT therefore does have the capacity to cause skin sensitisation in human models and also animal models as demonstrated by the contact hypersensitivity study in the guinea pig. Medical statements from a Corporate Medical Director and an Occupational Physician are available, and have been submitted. These medical statements make clear the fact that workers identified as 'at risk' from handling isothiazolones are monitored routinely and thoroughly within the workplace. Clearly, those most at risk in the population are those working in the manufacture of isothiazolones, with potential exposure to pure material on a daily basis. Despite the continuous potential exposure, very few cases of skin sensitisation within the workplace have occurred, indicative of high working practices. No cases of inhalation sensitisation are reported. **Skin Sensitisation: Summary** BIT has been demonstrated in both animal and human studies to have the capacity to cause skin sensitisation. Corresponding EU classification is as 'Irritant', with assignation of the hazard symbol 'Xi' and risk phrase 'R43 May cause sensitization by skin contact', in conformity with BIT's classification in Annex I to the Dangerous Substances Directive 67/54/EEC as amended. Additionally, classification of preparations containing BIT as 'Xi' and 'R43' is required when BIT concentration is ≥ 0.05%.	
Undertaking of intended data submission [ ]		
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	*November 2008*	
Evaluation of applicant's justification	*Applicant's justification is accepted.*	
Conclusion	*Applicant's summary of mammalian toxicology and conclusions are adopted.*	
Remarks		