BSL BIOSERVICE
Scientific Laboratories GmbH
Behringstr. 6/8
82152 Planegg
Germany

Phone +49 (0)89 899 650-0
Fax +49 (0)89 899 650-11

Test for Sensitisation
(Local Lymph Node Assay - LLNA)
with
Ammonium Niobium Oxalate

Report

Version: Final

Study Completion Date: 27 MAY 2014

BSL BIOSERVICE Study No.: 136509

Sponsor:

CBMM Europe BV
WTC H-Tower
Zuidplein 96
1077 XV Amsterdam
The Netherlands

1. Copy of the GLP Certificate

Bayerisches Landesamt für
Gesundheit und Lebensmittelsicherheit

GLP-Bescheinigung/Statement of GLP Compliance
(gemäß/according to § 19b Abs. 1 Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 2004/9/EG wurde durchgeführt in:

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/EC at:

☒ Prüfeinrichtung/Test facility ☐ Prüfstandort/Test site

BSL BIOSERVICE
SCIENTIFIC LABORATORIES GMBH
BEHRINGSTRAßE 6-8
82152 PLANEGG

(Unverwechselbare Bezeichnung und Adresse/Unequivocal name and address)

Prüfungen nach Kategorien/Areas of Expertise
(gemäß/according ChemVwV-GLP Nr. 5.3/OECD guidance)

Kategorie 2
Kategorie 3
Kategorie 9*
**Mikrobiologische Sicherheitsprüfungen; Wirksamkeitsprüfungen an Zellkulturen*

Datum der Inspektion/Date of Inspection
(Tag Monat Jahr/day month year)
03.- 04.07.2012

Die/Der genannte Prüfeinrichtung/Prüfstandort befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

The above mentioned test facility/test site is included in the national GLP Compliance Programme and is inspected on a regular basis.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung/diesem Prüfstandort die oben genannten Prüfungen unter Einhaltung der GLP-Grundsätze durchgeführt werden können.

Based on the inspection report it can be confirmed, that this test facility/test site is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Erlangen, 22.01.2013

Dr. Peter Franke
Leitender Regierungsdirektor

2. Contents

3. List of Tables

4. Preface

4.1. Abbreviations

^{3}HTdR	tritiated thymidine
Art.	Artikel *(Article)*
BGBl.	Bundesgesetzblatt *(Federal Law Gazette)*
Dipl.-Biol.	Diplom Biologe *(Biology Diploma)*
DMSO	dimethyl sulphoxide
DPM	disintegration per minute
EC	European Commission
EC3	estimated concentration of the test item required to produce a 3-fold stimulation of draining lymph node cell proliferation compared with concurrent controls
EPA	Environmental Protection Agency
GLP	Good Laboratory Practice
GmbH	Gesellschaft mit beschränkter Haftung *(company with limited liability)*
IVC	individually ventilated cages
LLNA	Local Lymph Node Assay
No.	number
Nr.	Nummer *(number)*
OECD	Organisation for Economic Cooperation and Development
OPPTS	Office of Prevention, Pesticides and Toxic Substances
PBS	phosphate buffered saline
QA	Quality Assurance
QAU	Quality Assurance Unit
SOP	Standard Operating Procedure
SPF	specific-pathogen free
TCA	trichloroacetic acid
v/v	volume per volume
w/v	weight per volume

4.2. General

Sponsor:	CBMM Europe BV WTC H-Tower Zuidplein 96 1077 XV Amsterdam The Netherlands
Study Monitor:	Dr. Claudia Schäfer Dr. Knoell Consult GmbH Marie-Curie-Str. 8 51377 Leverkusen Germany
Test Facility:	BSL BIOSERVICE Scientific Laboratories GmbH Behringstraße 6/8 82152 Planegg Germany
BSL BIOSERVICE Study No.:	136509
Test Item:	Ammonium Niobium Oxalate
Title:	Test for Sensitisation (Local Lymph Node Assay - LLNA) with Ammonium Niobium Oxalate

4.3. Project Staff

Study Director:	Dr. Sandra Schmid
Management:	Dr. Wolfram Riedel Dr. Angela Lutterbach Dr. Katrin Witschital
Head of Quality Assurance Unit:	Dipl.-Biol. Uwe Hamann

4.4. Schedule

Arrival of the Test Item:	21 May 2012
Study Initiation Date:	24 January 2014
Experimental Starting Date:	05 February 2014
Experimental Completion Date:	24 February 2014

5. Quality Assurance

5.1. GLP Compliance

This study was conducted to comply with:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Appendix 1 to § 19a as amended and promulgated on August 28, 2013 (BGBl. I S. 3498) [1].

Konsens-Dokument der Bund-Länder-Arbeitsgruppe Gute Laborpraxis ("Consensus Document of the National and Länder Working Party on Good Laboratory Practice") on the archiving and storage of records and materials, 5 May 1998 [2].

OECD Principles of Good Laboratory Practice (as revised in 1997); OECD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring - Number 1. Environment Directorate, Organisation for Economic Co-operation and Development, Paris 1998 [3].

This study was assessed for compliance with the study plan and the Standard Operating Procedures of BSL BIOSERVICE. The study and/or the test facility were periodically inspected by the Quality Assurance Unit according to the corresponding SOPs. These inspections and audits were carried out by the Quality Assurance Unit, personnel independent of staff involved in the study. A signed quality assurance statement, listing all performed audits, is included in the report.

5.2. Guidelines

This study followed the procedures indicated by internal BSL BIOSERVICE SOPs and the following internationally accepted guidelines and recommendations:

OECD Guidelines for Testing of Chemicals, number 429, "Skin Sensitisation: Local Lymph Node Assay" (adopted: July 22, 2010) [4]

Commission Regulation (EC) No. 440/2008, L 142, Annex Part B, Method B.42, 30 May 2008 [5]

EPA Health Effects Test Guidelines, OPPTS 870.2600 "Skin Sensitization", EPA 712-C-03-197, March 2003 [6]

5.3. Archiving

The records, materials and specimen will be stored according to the GLP regulations for a period of 15 years.

The following records have to be stored according to the GLP regulations:

A copy of the final report, the study plan and a documentation of all raw data generated during the conduct of the study (documentation forms as well as any other notes of raw data, printouts of instruments and computers) and the correspondence with the sponsor concerning the study. Any document relating to the study will be discarded only with the prior consent of the sponsor.

The following materials and samples have to be stored according to the period of time specified in the GLP regulations:

A retain sample of the test item will be archived according to the GLP regulations, if possible, and will be discarded without the sponsor's prior consent.

Other materials and specimen have to be stored according to the GLP regulations and disposed after the respective archiving period with the sponsor's prior consent.

Unless otherwise agreed in writing, the remaining test item will be discarded three months after the release of the report.

6. Statement of Compliance

BSL BIOSERVICE Study No.:	136509
Test Item:	Ammonium Niobium Oxalate
Title:	Test for Sensitisation (Local Lymph Node Assay - LLNA) with Ammonium Niobium Oxalate
Study Director:	Dr. Sandra Schmid

This study performed in the test facility BSL BIOSERVICE Scientific Laboratories GmbH was conducted in compliance with Good Laboratory Practice Regulations:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Appendix 1 to § 19a as amended and promulgated on August 28, 2013 (BGBl. I S. 3498) [1].

Konsens-Dokument der Bund-Länder-Arbeitsgruppe Gute Laborpraxis ("Consensus Document of the National and Länder Working Party on Good Laboratory Practice") on the archiving and storage of records and materials, 5 May 1998 [2].

"OECD Principles of Good Laboratory Practice (as revised in 1997)", Paris 1998 [3].

There were no circumstances that may have affected the quality or integrity of the study.

Study Director: Dr. Sandra Schmid

.....

Date: 27 May 2014

This statement does not include the prescreen test and the positive-control test.

7. Statement of the Quality Assurance Unit

BSL BIOSERVICE

Study No.:	136509
Test Item:	Ammonium Niobium Oxalate
Title:	Test for Sensitisation (Local Lymph Node Assay - LLNA) with Ammonium Niobium Oxalate
Study Director:	Dr. Sandra Schmid

This report and the conduct of this study were inspected by the Quality Assurance Unit on the following dates:

Phases of QAU Inspections	Dates of QAU Inspections	Dates of Reports to the Study Director and Management
Audit Final Study Plan:	23 January 2014	23 January 2014
Audit Experimental Phase (study-based):	17 February 2014	17 February 2014
Audit Final Report:	27 MAY 2014	27 MAY 2014

This report reflects the raw data.

Member of the
Quality Assurance Unit:

.....
Print Name: Dipl.-Ing. Gernot Zach

Date: 27 May 2014

This statement does not include the prescreen test and the positive-control test.

8. Summary

On the basis of the test results given below and in conformity with the criteria given in Commission Regulation (EU) No 286/2011 [7] the substance should be:

classified into sub-category 1A	☐
classified into sub-category 1B	☒
unclassified	☐

On the basis of the test results given below and in conformity with the criteria given in GHS (Globally Harmonized Classification System) [8] the substance should be:

classified into sub-category 1A	☐
classified into sub-category 1B	☒
unclassified	☐

Based on the results of the prescreen test the test item was assessed for sensitising properties at concentrations of 17%, 33% and 67% (w/v), each diluted with DMSO (dimethyl sulfoxide).

All animals survived throughout the test period.

One animal of the test group treated with the test item at a concentration of 33% showed bodyweight loss, hypothermia, dehydration, piloerection and lymph nodes with a small size. All other test group animals showed enlarged lymph nodes. Eschar was observed in all animals of the test group.

Species/strain:	Mice, CBA/CaOlaHsd
Number of animals:	20/main test
Vehicle:	DMSO (dimethyl sulfoxide)

8.1. Summary Results

Three of the three tested concentrations exceeded the stimulation index of 3.

The stimulation index at a concentration of	17%	was	**5.0**
The stimulation index at a concentration of	33%	was	**6.0**
The stimulation index at a concentration of	67%	was	**8.9**

8.2. Conclusion

The EC3 value (estimated by linear interpolation) was calculated to be at a test item concentration of 4.51%.

Consequently, according to OECD 429 [4] solutions or preparations containing more than 4.51% Ammonium Niobium Oxalate are expected to have a stimulation index of >3 and are therefore considered to be dermal sensitisers.

According to Commission Regulation (EU) No 286/2011 [7] as well as GHS (Globally Harmonized Classification System) [8] the test item Ammonium Niobium Oxalate has obligatory labelling requirement for skin sensitisation and is classified into Category 1B.

For details of the classification criteria see *Evaluation of Results*.

9. Introduction

9.1. Justification for the Selection of the Test System

The LLNA has been developed as an alternative method for the identification of skin sensitising test items and measures the proliferation of lymphocytes isolated from lymph nodes (auricular lymph nodes) draining the site of exposure (dorsal aspect of the ears) in mice.

Lymphocyte proliferation is measured by determining the incorporation of ^{3}H-methyl thymidine.

9.2. Justification for the Selection of the Test Method

No validated *in vitro* method is available for assessing sensitisation potential.

10. Materials and Methods

10.1. Characterisation of the Test Item

The identity of the test item was inspected upon delivery at the test facility (e.g. test item name, batch no. and additional data were compared with the label) based on the following specifications provided by the sponsor.

Name:	Ammonium Niobium Oxalate
Batch No.:	AD/4663
Chemical Name:	Reaction mass of ammonium diaqua[bis(oxalate)]oxoniobate(1-)hydrate and ammonium hydrogen oxalate oxalic acid (1:1:1) dehydrate
Purity:	≥ 96%
Physical State:	powder
Colour:	white
Storage Conditions:	at room temperature
Expiry Date:	25 March 2015
Safety Precautions:	The routine hygienic procedures were sufficient to assure personnel health and safety.

10.2. Vehicle

Due to the solubility properties of the test item the DMSO (Dimethyl sulfoxide, AppliChem, lot no. 2P003474, expiry date: 07/2014) was used.

10.3. Preparation of the Test Item

Based on the results observed in the prescreen test the following test item concentrations were selected for the main study:

17%, 33% and 67% (w/v)

The preparations were made immediately prior to each dosing.

10.4. Prescreen Test

Before the initiation of the prescreen test, a solubility test was performed to define the vehicle and the maximum concentration which is technically applicable to the animals.

The maximum technically applicable concentration of the test item was found to be 67% in the vehicle (see 10.2).

In order to determine the highest tolerated and not excessively irritant test concentration a prescreen test was performed which was conducted under the same conditions as the main study, except there was no assessment of lymph node proliferation. The mice were observed daily for any clinical signs of systemic toxicity or local irritation at the application site. Body weights were recorded pre-test and prior to termination. Both ears were

observed for erythema and scored according to Table 1. Ear thickness measurements were performed on day 1 (pre-dose), day 3 (approximately 48 hours after the first dose) and day 6. Excessive local irritation was indicated by an erythema score ≥ 3 and/or ear swelling of ≥ 25%.

Table 1: Erythema Scores

Erythema and Eschar Formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beef redness) to eschar formation preventing grading of erythema	4

Two animals were treated by topical application with the test item on three consecutive days at a concentration of 67% (diluted with DMSO) to the entire dorsal surface of each ear.

One further animal was treated with 100% DMSO and served as negative control.

Immediately before the first application, approximately 48 hours after the first application and shortly before sacrificing the thickness of both ears of all animals was measured (see Table 2).

Table 2: Ear Thickness (mm) – Prescreen Test

Group	Animal No.	Measurement of Ear Thickness (mm)					
		Day 1		Day 3		Day 6	
		left	right	left	right	left	right
Ammonium Niobium Oxalate 67% in DMSO	1	0.19	0.18	0.18	0.18	0.18	0.18
Ammonium Niobium Oxalate 67% in DMSO	2	0.18	0.18	0.17	0.17	0.17	0.16
Negative Control 100% DMSO	3	0.18	0.18	0.17	0.18	0.18	0.18

During this period also all clinical signs were recorded.

Cageside observations included spontaneous activity, lethargy, recumbent position, convulsions, tremors, apnoea, asphyxia, vocalisation, diarrhoea, changes in the skin and fur, eyes and mucous membranes (salivation, discharge).

Neither signs of systemic toxicity nor signs of irritation at any application site could be detected in any animal.

Animal no. 2 showed a weight loss of 4 g. All other animals showed the expected weight development, which includes a weight loss of up to 2 g throughout the duration of the prescreen test (see Table 3).

Table 3: Absolute Body Weights (g) – Prescreen Test

Group	Animal No.	Body Weights (g)		Weight Gain (g)
		Start of Study	End of Study	
Ammonium Niobium Oxalate 67% in DMSO	**1**	23	23	**0**
Ammonium Niobium Oxalate 67% in DMSO	**2**	22	18	**-4**
Negative Control 100% DMSO	**5**	23	23	**0**

10.5. Control

DMSO (see 10.2) was used as vehicle and served as negative control.

10.6. Other Materials

^{3}H-methyl thymidine (TRK 300, 20 Ci/mmol; PerkinElmer, lot no. 201310E), diluted to a working concentration of 80 µCi/mL.

Phosphate buffered saline (PBS), BSL BIOSERVICE, lot no. 24122013, expiry date: 07/2014.

Trichloroacetic acid (TCA), Sigma, lot no. BCBH2990V, expiry date: 02/2014.

10.7. Test System

Species/strain:	CBA/CaOlaHsD mice
Source:	Harlan Laboratories GmbH, 5800 AN Venray, The Netherlands
Sex:	female (nulliparous and non-pregnant)
Age at the beginning of the study:	9-10 weeks
Number of animals:	5 mice / group 3 mice / prescreen test

The animals were derived from a controlled full-barrier maintained breeding system (SPF). According to Art. 9.2, No. 7 of the German Act on Animal Welfare [9] the animals are bred for experimental purposes.

10.7.1. Housing and Feeding Conditions

- Full barrier in an air-conditioned room
- Temperature: 22 ± 3 °C
- Relative humidity: 55 ± 10%
- Artificial light, sequence being 12 hours light, 12 hours dark
- Air change: at least 10 x / hour
- Free access to Altromin 1324 maintenance diet for rats and mice lot no. 0801)
- Free access to tap water, sulphur acidified to a pH value of approx. 2.8 (drinking water, municipal residue control, microbiological controls at regular intervals)
- The animals were kept in groups of 5 animals in IVC cages, type II L, polysulphone cages on Altromin saw fibre bedding (lot no. 131113)
- Certificates of food, water and bedding are filed at BSL BIOSERVICE
- Adequate acclimatisation period (at least five days) under laboratory conditions

10.8. Preparation of the Animals

The animals were randomly selected.

Identification was ensured by cage number and individual marking (tail).

10.9. Clinical Observation

Prior to the application and once a day thereafter all animals were observed in order to detect signs of toxicity, including dermal irritation at site of application.

10.10. Weight Assessment

The animals were weighed prior to the application and at the end of the test period (prior to the treatment with ^{3}HTdR).

10.11. Dose Groups

3 test groups (3 different concentrations) and 1 negative control group (vehicle) were tested.

10.12. Test Regime

Topical Application

Each mouse was treated by topical application of 25 µL of the selected solution to the entire dorsal surface of each ear.

Topical applications were performed once daily over three consecutive days.

Administration of ^{3}H-Methyl Thymidine

Five days after the first topical application all mice were dosed with 20 µCi ^{3}H-methyl thymidine by intravenous injection (tail vein) of 250 µL of ^{3}H-methyl thymidine, diluted to a working concentration of 80 µCi/mL.

Preparation of Cell Suspension

Approximately 5 hours after the injection of ^{3}H-methyl thymidine all mice were sacrificed by cervical dislocation. The draining auricular lymph nodes were excised, individually pooled for each animal (2 lymph nodes per animal) and collected in phosphate buffered saline (PBS). A single cell suspension of pooled lymph node cells was prepared by gentle mechanical disaggregation through polyamide gauze (200 mesh size). After washing the gauze with PBS the cell suspension was pelleted in a centrifuge. The supernatant was discarded and the pellets were resuspended with PBS. This washing procedure was repeated.

After the final wash each pellet was resuspended in approx. 1 mL 5% TCA at approx. 4° C for approximately 18 hours for precipitation of macromolecules. Each precipitate was once washed again, resuspended in 1 mL 5% TCA and 7 mL scintillation fluid was added. Then this solution was transferred into scintillation vials and stored at room temperature overnight.

Determination of Incorporated ^{3}H -Methyl Thymidine

The ^{3}H-methyl thymidine – incorporation was measured in a ß-counter and expressed as the number of disintegrations per minute (DPM). Similarly, background ^{3}H-methyl thymidine levels were also measured (5% TCA). Determination of radioactivity was performed individually for each animal.

10.13. Reliability Check

The recent reliability check was performed in November 2013. The raw data of this study are kept in the BSL archives (BSL Project ID 134702 O).

Positive-control substance:	P-Phenylenediamine (CAS 106-50-3, Sigma, purity > 98%; Lot No.: SLBC7171V) 1%
Vehicle:	DMSO (dimethyl sulfoxide)
Species/strain:	healthy CBA/CaOlaHsd mice
Source:	Harlan Laboratories GmbH, 5800 AN Venray, The Netherlands
Concentrations:	1% on three consecutive days

Table 4: Radioactive Determination of the Positive-Control Group of the Recent Study

POS	CPM	Test Item	Conc. [%]	Animal number	DPM	DPM-mean back-ground	DPM/ Node	Stimu-lation Index
46	1962.0	**Negative**		46	5067.0	5048.8	2524.4	
47	1425.0	**Control**		47	3653.0	3634.8	1817.4	
48	1137.0			48	2972.0	2953.8	1476.9	
49	1625.0			49	4230.0	4211.8	2105.9	
50	1160.0			50	3088.0	3069.8	1534.9	
MV	**1461.8**			**MV**	**3802.0**	**3783.8**	**1891.9**	**1.0**
SD	**308.1**			**SD**	**775.2**	**775.2**	**387.6**	
51	14555.0	**Phenylene-**	**1**	51	38249.0	38230.8	19115.4	10.1
52	12373.0	**diamine**		52	33102.0	33083.8	16541.9	8.7
53	14562.0			53	37947.0	37928.8	18964.4	10.0
54	13419.0			54	35188.0	35169.8	17584.9	9.3
55	9471*			55	24783*	n.d.	n.d.	n.d.
MV	**13727.3**			**MV**	**36121.5**	**36103.3**	**18051.7**	**9.5**
SD	**909.8**			**SD**	**2112.3**	**2112.3**	**1056.2**	**0.6**
56	6.0	**Background**			16.0			
57	11.0	**Szinti and**			29.0			
58	7.0	**TCA**			19.0			
59	5.0				13.0			
60	5.0				14.0			
MV	**6.8**			**MV**	**18.2**	**0.0**	**0.0**	**0.0**
SD	**2.2**			**SD**	**5.8**			

If not noted individually, the results include both lymph nodes of an animal.

POS = position in counter; CPM = counts per minute; Conc. = concentration; DPM = disintegrations per minute; SD = standard deviation; MV = mean value; Szinti = scintillation fluid; TCA = trichloroacetic acid

10.14. Evaluation of Results

The proliferative response of lymph node cells was expressed as the number of radioactive disintegrations per minute per lymph node (DPM/NODE) and as the ratio of ^{3}H-methyl thymidine - incorporation into lymph node cells of test group animals relative to that recorded for control group animals (STIMULATION INDEX). Before DPM/NODE values were determined, background values were subtracted.

EC3 values, calculated concentrations which induce stimulation indices of three, are determined by linear interpolation, EC3=c+[(3-d)/(b-d)]x(a-c), between two points of the stimulation indices axis, one above (a,b) and one below (c,d) the stimulation index of three [10], [12]. If all measured points are above or below the stimulation index of three, no EC3 value can be stated.

In certain situations where the dose response does not incorporate a data point lying below the SI value of three, provided the data are of good quality (relatively close to an SI of three and evidence of a dose response), an EC3 value may be estimated by using the two doses closest to the SI value of three. The EC3 value is estimated by log-linear interpolation between these two points on a plane where the x-axis represents the dose level and the y-axis represents the SI. The point with the higher SI is denoted (a,b) and the point with the lower SI is denoted (c,d). The formula for the EC3 estimate is as follows: EC3=2^{(log2(c)+(3-d)/(b-d)*[(log2(a)-log2(c)]}, by log-transforming the doses, EC3 estimates will never fall below zero [11], [12].

A substance is regarded as a 'sensitiser' in the LLNA if at least one concentration of the test item results in a 3-fold or greater increase in ^{3}H-methyl thymidine - incorporation into lymph node cells of the test group animals, relative to that recorded for the lymph nodes of control group animals (**Stimulation Index equal to or greater than 3.0**).

On the basis of the test results, the test substance may be classified into one of the following categories in conformity with the criteria given in **Commission Regulation (EU) No 286/2011** [7] as well as in **GHS - Globally Harmonised System of Classification and Labelling of Chemicals, fifth revised edition, 2013** [8]:

Skin sensitiser

Category 1:

A substance is classified as a skin sensitiser

a) if there is evidence in humans that the substance can lead to sensitisation by skin contact in a substantial number of persons, or

b) if there are positive results from an appropriate animal test.

WARNING, exclamation mark. May cause an allergic skin reaction.

Sub-category 1A:

Substances showing a high frequency of occurrence in humans and/or a high potency in animals can be presumed to have the potential to produce significant sensitisation in humans. Severity of reaction may also be considered.

EC3 value ≤ 2%

WARNING, exclamation mark. May cause an allergic skin reaction.

Sub-category 1B:

Substances showing a low to moderate frequency of occurrence in humans and/or a low to moderate potency in animals can be presumed to have the potential to produce sensitisation in humans. Severity of reaction may also be considered.

EC3 value > 2%

WARNING, exclamation mark. May cause an allergic skin reaction.

11. Deviations from the Study Plan

There was the following deviation from the study plan:

Concerning:

Evaluation of Results, study plan, p. 13

Before:

EC3 values, calculated concentrations which induce stimulation indices of three, will be determined by linear interpolation between two points of the stimulation indices axis, one above and one below the stimulation index of three. If all measured points are above or below the stimulation index of three, no EC3 value can be stated.

New:

EC3 values, calculated concentrations which induce stimulation indices of three, are determined by linear interpolation, EC3=c+[(3-d)/(b-d)]x(a c), between two points of the stimulation indices axis, one above (a,b) and one below (c,d) the stimulation index of three [10], [12]. If all measured points are above or below the stimulation index of three, no EC3 value can be stated.

In certain situations where the dose response does not incorporate a data point lying below the SI value of three, provided the data are of good quality (relatively close to an SI of three and evidence of a dose response), an EC3 value may be estimated by using the two doses closest to the SI value of three. The EC3 value is estimated by log-linear interpolation between these two points on a plane where the x-axis represents the dose level and the y-axis represents the SI. The point with the higher SI is denoted (a,b) and the point with the lower SI is denoted (c,d). The formula for the EC3 estimate is as follows: EC3=2^{(log2(c)+(3-d)/(b-d)*[(log2(a)-log2(c)]}, by log-transforming the doses, EC3 estimates will never fall below zero [11], [12].

Reason:

All stimulation indices above three.

This deviation did not influence the quality or integrity of the present study.

12. Results

Three of the three tested concentrations exceeded the stimulation index of 3.

The stimulation index at a concentration of 17% was **5.0**

The stimulation index at a concentration of 33% was **6.0**

The stimulation index at a concentration of 67% was **8.9**

For individual data see Table 5 in the appendix.

All animals survived throughout the test period. On day 6 animal no. 9 of the test group (test item concentration 33%) showed bodyweight loss, hypothermia, dehydration, piloerection and lymph nodes with a small size. All other test group animals showed enlarged lymph nodes. Eschar was observed in all animals of the test group on day 6.
As only for one animal in the test group (test item concentration 33%) clinical findings were recorded, a clear test item relation cannot be concluded.

For individual data see Table 7 in the appendix.

12.1. Body Weight Development

One animal (animal no. 9) treated with a test item concentration of 33% showed a weight loss of 4 g.

All other animals showed the expected weight development, which includes a weight loss of up to 2 g throughout the study.

For individual data see Table 6 in the appendix.

13. Conclusion

The EC3 value (estimated by linear interpolation) was calculated to be at a test item concentration of 4.51%.

Consequently, according to OECD 429 [4] solutions or preparations containing more than 4.51% Ammonium Niobium Oxalate are expected to have a stimulation index of >3 and are therefore considered to be dermal sensitisers.

According to Commission Regulation (EU) No 286/2011 [7] as well as GHS (Globally Harmonized Classification System) [8] the test item Ammonium Niobium Oxalate has obligatory labelling requirement for skin sensitisation and is classified into Category 1B.

For details of the classification criteria see *Evaluation of Results*.

14. Distribution of the Report

1 original (paper):	Sponsor
1 copy (paper):	BSL BIOSERVICE
1 copy (electronic):	Sponsor

15. References

15.1. Internal BSL BIOSERVICE SOPs

Standard Operating Procedures (SOPs), No. 11-3-3

15.2. Literature and Guidelines

[1] Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Appendix 1 to § 19a as amended and promulgated on August 28, 2013 (BGBl. I S. 3498)

[2] Konsens-Dokument der Bund-Länder-Arbeitsgruppe Gute Laborpraxis ("Consensus Document of the National and Länder Working Party on Good Laboratory Practice") on the archiving and storage of records and materials, 5 May 1998

[3] OECD Principles of Good Laboratory Practice (as revised in 1997); OECD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring - Number 1. Environment Directorate, Organisation for Economic Co-operation and Development, Paris 1998

[4] OECD Guidelines for Testing of Chemicals, number 429, "Skin Sensitisation: Local Lymph Node Assay" (adopted: July 22, 2010)

[5] Commission Regulation (EC) No 440/2008, L 142, Annex Part B, Method B.42 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No. 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH)

[6] EPA Health Effects Test Guidelines, OPPTS 870.2600 Skin Sensitization, EPA 712-C-03-197, March 2003, United States, Environmental Protection Agency, Prevention, Pesticides and Toxic Substances (7101)

[7] Commission Regulation (EU) No 286/2011 of 10 March 2011, amending, for the purposes of its adaptation to technical and scientific progress, Regulation (EC) No 1272/2008 of the European Parliament and of the Council on classification, labelling and packaging of substances and mixtures. Official Journal of the European Union, L 83/1, 30 March 2011

[8] GHS - Globally Harmonized System of Classification and Labelling of Chemicals. Fifth revised edition, United Nations. New York / Geneva, 2013

[9] German Animal Welfare Act, Art. 9.2, No. 7 (Deutsches Tierschutzgesetz, 24. Juli 1972 (BGBl. I S. 1277), Inkrafttreten der letzten Änderung: 13. Juli 2013, (Art. 2 ÄndG vom 04. Juli 2013)

[10] Basketter, D.A., Lea, L.J., Dickens, A., Briggs, D., Pate, I., Dearman, R. and Kimber, I. (1999) A comparison of statistical approaches to the derivation of EC3 values from local lymph node assay dose responses. Journal of Applied Toxicology, 19,261-266

[11] QMRF (Q16-10-22-170), „QSAR for classification of skin sensitisation in FQSARModel", Feb 16, 2010

[12] ICCVAM. 2011. ICCVAM Test Method Evaluation Report: Usefulness and Limitations of the Murine Local Lymph Node Assay for Potency Categorization of Chemicals Causing Allergic Contact Dermatitis in Humans. NIH Publication No. 11-7709. Research Triangle Park, NC:National Institute of Environmental Health Sciences.

16. Appendix

16.1. Appendix 1: Individual Data

Table 5: Radioactive Determination of the Test Substance Groups

POS	CPM	Test Item	Conc. [%]	Animal number	DPM	DPM-mean back-ground	DPM/ Node	Stimu-lation Index
66	1012.0	Negative Control	100	16	2673.0	2657.4	1328.7	
67	1334.0			17	3554.0	3538.4	1769.2	
68	737.0			18	1931.0	1915.4	957.7	
69	1303.0			19	3414.0	3398.4	1699.2	
70	1482.0			20	3900.0	3884.4	1942.2	
MV	**1173.6**			**MV**	**3094.4**	**3078.8**	**1539.4**	**1.0**
SD	**266.2**			**SD**	**706.2**	**706.2**	**353.1**	
51	3566.0	Ammonium Niobium Oxalate in DMSO	17	1	9352.0	9336.4	4668.2	3.0
52	5528.0			2	14601.0	14585.4	7292.7	4.7
53	8325.0			3	21971.0	21955.4	10977.7	7.1
54	6898.0			4	18192.0	18176.4	9088.2	5.9
55	4916.0			5	13031.0	13015.4	6507.7	4.2
MV	**5846.6**			**MV**	**15429.4**	**15413.8**	**7706.9**	**5.0**
SD	**1638.1**			**SD**	**4331.2**	**4331.2**	**2165.6**	**1.4**
56	6094.0	Ammonium Niobium Oxalate in DMSO	33	6	16040.0	16024.4	8012.2	5.2
57	7454.0			7	19542.0	19526.4	9763.2	6.3
58	6605.0			8	17448.0	17432.4	8716.2	5.7
59	391*			9	1015*	n.d.	n.d.	n.d.
60	7774.0			10	20669.0	20653.4	10326.7	6.7
MV	**6981.8**			**MV**	**18424.8**	**18409.2**	**9204.6**	**6.0**
SD	**667.2**			**SD**	**1797.6**	**1797.6**	**898.8**	**0.6**
61	16034.0	Ammonium Niobium Oxalate in DMSO	67	11	42704.0	42688.4	21344.2	13.9
62	8341.0			12	21755.0	21739.4	10869.7	7.1
63	12362.0			13	33252.0	33236.4	16618.2	10.8
64	5230.0			14	13824.0	13808.4	6904.2	4.5
65	9641.0			15	25374.0	25358.4	12679.2	8.2
MV	**10321.6**			**MV**	**27381.8**	**27366.2**	**13683.1**	**8.9**
SD	**3663.5**			**SD**	**9886.7**	**9886.7**	**4943.4**	**3.2**
76	7.0	Background Szinti and TCA			18.0			
77	6.0				14.0			
78	6.0				18.0			
79	5.0				12.0			
80	6.0				16.0			
MV	**6.0**			**MV**	**15.6**	**0.0**	**0.0**	**0.0**
SD	**0.6**			**SD**	**2.3**			

* = outlier, failed Grubbs, Nalimov and Dixon; n.d. = not determined

If not noted individually, the results include both lymph nodes of an animal.

POS = position in counter; CPM = counts per minute; Conc. = concentration; DPM = disintegrations per minute; SD = standard deviation; MV = mean value; Szinti = scintillation fluid; TCA = trichloroacetic acid

Table 6: Absolute Body Weights in g

Group	Animal No.	Body Weights (g)		Weight Gain
		Start of Study	End of Study	
Ammonium Niobium Oxalate 17% in DMSO	1	21	21	0
	2	21	22	1
	3	21	20	-1
	4	22	24	2
	5	21	22	1
Ammonium Niobium Oxalate 33% in DMSO	6	20	21	1
	7	19	21	2
	8	20	22	2
	9	19	15	-4
	10	22	22	0
Ammonium Niobium Oxalate 67% in DMSO	11	21	19	-2
	12	23	22	-1
	13	21	21	0
	14	23	22	-1
	15	22	20	-2
Negative Control 100% DMSO	16	23	22	-1
	17	19	21	2
	18	20	22	2
	19	22	24	2
	20	23	23	0

Table 7: Clinical Observation

Time of Observation	Systemic Effects	Local Effects
Group 1, animals no. 1 – 5 / test item at a concentration of 17% in DMSO		
Day 1	nsf	nsf
Day 2	nsf	nsf
Day 3	nsf	nsf
Day 4	nsf	nsf
Day 5	nsf	nsf
Day 6	nsf	eschar
Group 2, animals no. 6 – 10 / test item at a concentration of 33% in DMSO		
Day 1	nsf	nsf
Day 2	nsf	nsf
Day 3	nsf	nsf
Day 4	nsf	nsf
Day 5	nsf	nsf
Day 6	nsf	hypothermia, dehydration, piloerection*, eschar
Group 3, animals no. 11 – 15 / test item at a concentration of 67% in DMSO		
Day 1	nsf	nsf
Day 2	nsf	nsf
Day 3	nsf	nsf
Day 4	nsf	nsf
Day 5	nsf	nsf
Day 6	nsf	eschar
Group 4, animals no. 16 – 20 / negative control DMSO		
Day 1	nsf	nsf
Day 2	nsf	nsf
Day 3	nsf	nsf
Day 4	nsf	nsf
Day 5	nsf	nsf
Day 6	nsf	nsf

nsf = no specific findings, * = observed in animal no. 9

16.2. Appendix 2: Certificate of Analysis

COMPANHIA BRASILEIRA DE METALURGIA E MINERAÇÃO
Córrego da Mata S/N - C.P. 08 - Araxá - Minas Gerais - Cep: 38.183-970 - Brasil
Phone: (55-34) 3669-3000 - Facsimile: (55-34) 3669-3300

CERTICATE OF ANALYSIS

		NUM.	DATE: 05/09/2012
PRODUCT: AMMONIUM NIOBIUM OXALATE	LOT: AD/4663	SIZING	QUANTITY: 15
MARK	CUSTOMER: BSL BIOSERVICE Scientific Laboratories GmbH		PACKAGING: 1/1

Element	Analysis
% Nb2O5	24.9
ppm Ca	<1
ppm Fe	39
ppm K	89
ppm Mg	<25
ppm Na	17
ppm Ta	122
ppm Ti	<1

Size Distribution	
Screen (mm)	(%) Analysis

Observation

Emitted by	Approved by
Leandro Oliveira Lima	Andreia Duarte Menezes Teixeira
Chemist	Lab. Manager