BIOSERVICE

SCIENTIFIC LABORATORIES GmbH

Screening for the Eye Irritancy Potential using the Chorioallantoic Membrane Assay with Niobium

Report

Version: Final

Date: 19 November 2009

BSL BIOSERVICE Study No.: 092570A

Sponsor:

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

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Amtsgericht München, HRB 109 770
Erfüllung und Gerichtsstand München
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GLP zertifiziert

Akkreditiert durch
Zentralstelle der Länder für Gesundheitsschutz bei Arzneimitteln und Medizinprodukten
ZLG-P-986.96.01

1. Copy of the GLP Certificate

BAYERISCHES LANDESAMT
FÜR GESUNDHEIT UND LEBENSMITTELSICHERHEIT,
LANDESINSTITUT FÜR ARBEITSSCHUTZ UND PRODUKTSICHERHEIT
Pfarrstraße 3 · 80538 München · Telefon (089) 21 84-0

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)

2 Prüfungen auf toxikologische Eigenschaften
3 Prüfungen auf mutagene Eigenschaften
9 Sonstige Prüfungen:
a) Mikrobiologische Sicherheitsprüfungen
b) Wirksamkeitsprüfungen an Zellkulturen

Datum der Inspektion/Date of Inspection
(Tag.Monat.Jahr/day.month.year)
16./17.09.2008

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.

München, 06.04.2009

Ritter
Leitender Gewerbedirektor

2. Contents

3. List of Tables and Figures

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4. Preface

4.1. Abbreviations

BGBl.	Bundesgesetzblatt
GLP	Good Laboratory Practice
OECD	Organisation of Economic Cooperation and Development
QA	Quality Assurance
QAU	Quality Assurance Unit
SOP	Standard Operating Procedures

4.2. General

Sponsor:	CBMM Europe BV WTC H- Tower Zuidplein 96 1077 XV, Amsterdam The Netherlands
Study Monitor:	Mr Jorge Davo CBMM Companhia Brasileira de Metalurgia e Mineração Córrego da Mata s/n 38183-903 Araxá - MG Brasil
Test Facility:	BSL BIOSERVICE Scientific Laboratories GmbH Behringstraße 6/8 82152 Planegg Germany
BSL BIOSERVICE Study No.:	092570A
Test Item:	Niobium
Title:	Screening for the Eye Irritancy Potential using the Chorioallantoic Membrane Assay with Niobium

4.3. Project Staff

Study Director:	Dr. Achim Albrecht
Deputy Study Director:	Dr. Daniela Stelter
Management:	Dr. Wolfram Riedel Dr. Angela Lutterbach
Head of Quality Assurance Unit:	Dipl.-Biol. Uwe Hamann

4.4. Schedule

Arrival of the Test Item:	20 July 2009
Date of Draft Study Plan:	30 July 2009
Date of Final Study Plan:	07 August 2009
Start of Experiment:	04 October 2009
End of Experiment:	14 October 2009
Date of Draft Report:	09 November 2009
Date of Final Report:	19 November 2009

5. Project Staff Signatures

Study Director

Dr. Achim Albrecht

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Date: 19 Nov 2009

Management

.....

Print Name: Dr. Angela Lutterbach

Date: 19 Nov 2009

6. Quality Assurance

6.1. 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 June 20, 2002 (BGBl. 1 Nr. 40 S. 2090), revised October 31, 2006 (BGBl. I Nr. 50 S. 2407).

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.

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.

6.2. Guidelines

There are no OECD and EC guidelines and recommendations available for this test system.

6.3. Archiving

The following records will be stored in the scientific archives of BSL BIOSERVICE Scientific Laboratories GmbH 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.

If test item is left, a sample will be stored according to the period fixed by the GLP Regulations. Samples that are unstable may be disposed of before that time. No raw data or material relating to the study will be discarded without the Sponsor's prior consent. Unless otherwise agreed upon, the remaining test item will be discarded three months after the release of the report.

7. Statement of Compliance

BSL BIOSERVICE-Study No.:	092570A
Test Item:	Niobium
Title:	Screening for the Eye Irritancy Potential using the Chorioallantoic Membrane Assay with Niobium
Study Director:	Dr. Achim Albrecht

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 June 20, 2002 (BGBl. Nr. 40 S. 2090), revised October 31, 2006 (BGBl. I Nr. 50 S. 2407).

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

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

Study Director: Dr. Achim Albrecht

Date: 03 Dec 2009

8. Statement of the Quality Assurance Unit

BSL BIOSERVICE-Study No.:	092570A
Test Item:	Niobium
Title:	Screening for the Eye Irritancy Potential using the Chorioallantoic Membrane Assay with Niobium
Study Director:	Dr. Achim Albrecht

This report was audited by the Quality Assurance unit and the conduct of this study was inspected 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:	12 August 2009	12 August 2009
Audit experimental Phase (Method Audit):	15 June 2009	15 June 2009
Audit Final Report:	27 November 2009	27 November 2009

This report reflects the raw data.

Member of the
Quality Assurance unit:

Ulrike Fuhrmann

Print Name: Dipl. oec. troph. Ulrike Fuhrmann

Date: 03. Dec. 2009

9. Summary

The eye irritancy potential of the test item was investigated in the chorioallantoic membrane assay.

The test item was applied after an extract preparation and tested as a 100% extract (extraction ratio: 1 g test item / 5 mL physiological saline 0.9% NaCl; extraction conditions: 37 ± 1 °C for 72 ± 2 h, under agitation) in order to find any irritancy potential:

The calculated mean irritation score was 0.

The test item was classified as non-irritant.

The positive and negative controls were within the historical control data range demonstrating the validity and sensitivity of the test.

9.1. Conclusions

According to the evaluation criteria the test item Niobium is classified as non-irritant to mucous membranes.

Under the conditions of the present study, it can be stated that no irritation can be expected if Niobium comes in contact with the eyes.

10. Aim of the Study

10.1. Justification for Selection of the Test System

This study was performed to assess the eye irritancy potential of the test item using the chorioallantoic membrane (CAM) of incubated hens' eggs.

The potential irritancy of compounds may be detected observing adverse changes that occur in the chorioallantoic membrane of the egg after exposure to test chemicals.

Chemicals are placed directly onto the chorioallantoic membrane of the hen's egg. Hens' eggs are rotated in an incubator for 9 d, after which any defective eggs are discarded. The shell around the air cell is removed and the inner membranes are extracted to reveal the chorioallantoic membrane. The test chemicals are added to the membrane and left in contact for 5 min. The membrane is examined for vascular damage and the time taken for injury to occur is recorded. Irritancy is scored according to the severity (grade of reaction at the time of first occurrence = s) and speed at which damage occurs.

10.2. Justification for Selection of the Test Method

The occurrence of vascular injury or coagulation in response to a compound is the basis for employing this technique as an indication of the potential of a chemical to damage mucous membranes (in particular the eye) in vivo.

To validate CAM-data, reference substances are tested in parallel to the test item.

11. Materials and Methods

11.1. Characterisation of the Test Item

The test item and the information concerning the test item were provided by the Sponsor. All data related to the test item are the responsibility of the Sponsor and have not been verified by the test facility.

Name:	Niobium
Product:	Niobium Metal (Nb)
CAS no.:	7440-03-1
Batch no.:	AD/4202
Chemical name:	niobium
Density:	~8.5 g / cm^3
Active components:	Nb - >98.5%
Colour:	silver grey metalic
Physical state:	solid
Storage:	at room temperature
Stability:	stable
Safety precautions:	Routine hygienic procedures were sufficient to assure personnel health and safety.

11.2. Preparation of the Test Item

The test item was applied after extract preparation.

11.3. Extract Preparation of the Test Item

Solvent:

Physiological saline 0.9% NaCl, B. Braun Melsungen, lot no. 9181A121, expiry date: March 2012

The extract preparation was performed according to guideline ISO 10993-12.

In total a ratio of 1 g of sample to 5 mL of extraction medium was used.

Extraction conditions: 37 ± 1 °C for 72 ± 2 h, under agitation.

The extract application was carried out on the same day.

11.4. Incubation of Eggs

White eggs (White Leghorn) were obtained freshly fertilised from a chicken breeding centre.

The eggs were candled and any defective eggs were discarded.

The eggs were placed onto incubator trays with the ends against one another. The trays were placed into the incubator, which automatically rotates the eggs once every 4 hours. An optimum temperature of 37.5 ± 1 °C and a relative humidity of 50 - 70% was maintained throughout the incubation period.

On day 9 the eggs were placed the large end upward without rotation, thus ensuring accessibility to the chorioallantoic membrane.

On day 10 the eggs were prepared for assaying.

11.5. Assay Preparation

The egg shell was removed from the eggs' air chamber.

The membrane was carefully moistened with physiological saline 0.9% NaCl (B. Braun Melsungen, lot no. 9181A121, expiry date: March 2012) solution at 37 °C.

The eggs were replaced into the incubator until start of assaying (maximum of 30 min between opening the eggs and starting the assay).

Standard solutions were freshly prepared (except NaOH – stock solution) in appropriate solvents at room temperature.

11.6. Assay Procedure

The egg was tabled out of the incubator, the NaCl solution was poured off and the membrane was carefully removed using tapered forceps. Eggs with injured blood vessels were discarded.

0.3 mL of the standard solution or the test item extract were added to the chorioallantoic membrane (CAM).

The reactions on the CAM were observed over a period of 5 min. monitoring the appearance of:

H: Haemorrhage (bleeding)

L: Vascular lysis (blood vessel disintegration)

C: Coagulation (protein denaturation intra- and extravascular)

11.7. Test Groups

6 eggs for the test item

2 eggs as negative controls treated with isotonic saline solution (physiological saline 0.9% NaCl) (B. Braun Melsungen, lot no. 9181A121, expiry date: March 2012)

4 eggs as positive controls: 2 eggs treated with NaOH (0.1 N)

2 eggs treated with SDS (1%)

11.8. Positive Control Substances:

Sodium hydroxide solution (0.1 N), NaOH, (Applichem, lot no. 7W008967, expiry date: January 2011) dissolved in aqua ad inj., (B. Braun Melsungen, Lot 7494A191, expiry date: November 2010)

Dodecyl sulfate sodium salt (1%), SDS, (Sigma, lot no. 098K0160, expiry date: 01 April 2010) dissolved in aqua ad inj., (B. Braun Melsungen, Lot 7494A191, expiry date: November 2010)

Validity: The CAM Assay is considered to be valid if the scores obtained with the positive control substances fall within the range of the reference control data:

NaOH (0.1 N)	IS = 15 ± 3
SDS (1%)	IS = 10 ± 2

11.9. Evaluation of Results

The time for each reaction to occur was recorded and an irritation score (IS) was calculated:

$$IS = [(301\text{-sec H})/300] \times 5 \times S + [(301\text{-sec L})/300] \times 7 \times S + [(301 - \text{sec C})/300] \times 9 \times S$$

sec = second of first occurence of reaction

H = Haemorrhage

L = Vascular Lysis

C = Coagulation

S = 0.1 if H, C, L is grade 1 (weak reaction)

S = 0.5 if H, C, L is grade 2 (moderate reaction)

S = 1 if H, C, L is grade 3 (strong reaction)

The mean score was calculated from irritation scores for each egg for each test group.

The results were evaluated according to table 1.

Table 1: Evaluation of the CAM Assay

Mean Score	*Evaluation*
0 - 1.9	not irritant
2.0 - 5.9	slight irritant
6.0 - 10.9	moderate irritant
11.0 - 21.0	severe irritant

12. Deviations from the Study Plan

There was no deviation from the study plan.

13. Results and Discussion

The eye irritancy potential of the test item was investigated in the chorioallantoic membrane assay.

The test item was applied after an extract preparation and tested as a 100% extract (extraction ratio: 1 g test item / 5 mL physiological saline 0.9% NaCl; extraction conditions: 37 ± 1 °C for 72 ± 2 h, under agitation) in order to find any irritancy potential:

The calculated mean irritation score was 0.

The test item was classified as non-irritant.

The positive and negative controls were within the historical control data range demonstrating the validity and sensitivity of the test.

13.1. Conclusions

According to the evaluation criteria the test item Niobium is classified as non-irritant to mucous membranes.

Under the conditions of the present study, it can be stated that no irritation can be expected if Niobium comes in contact with the eyes.

Table 2: Results of the Chorioallantoic Membrane Assay - Control

Table 2	**Results of the Chorioallantoic Membrane Assay**				092570A-D			
Test day: 14 October 2009								
Controls	Haemorrhage in sec.	S	Lysis in sec.	S	Coagulation in sec.	S	IS	**IS Mean**
1-NaOH	12	1	22	1	103	1	**17.27**	
2	13	1	25	1	110	1	**16.97**	
severe irritant								**17.12**
1-NaCl	0	0	0	0	0	0	**0.00**	
2	0	0	0	0	0	0	**0.00**	
not irritant								**0.00**
1-SDS 1%	35	1	100	1	220	0.5	**10.34**	
2	37	1	105	1	225	0.5	**10.11**	
moderate irritant								**10.23**

Table 3: Results of the Chorioallantoic Membrane Assay – Test Item

Table 3	**Results of the Chorioallantoic Membrane Assay**				092570A			
Test Day: 14 October 2009								
Test Item: Extract of Niobium	Haemorrhage in sec.	S	Lysis in sec.	S	Coagulation in sec.	S	IS	**IS Mean**
1-Code 1 / 100% extract	0	0	0	0	0	0	**0.00**	
2	0	0	0	0	0	0	**0.00**	
3	0	0	0	0	0	0	**0.00**	
4	0	0	0	0	0	0	**0.00**	
5	0	0	0	0	0	0	**0.00**	
6	0	0	0	0	0	0	**0.00**	
not irritant								**0.00**

14. Distribution of the Report

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

15. References

BSL BIOSERVICE, Standard Operating Procedures (SOP) No. 11-8-1

Helmrich, Ch. (1992)
Evaluierung von Ersatzmethoden für den Draize-Test am Kaninchenauge
Diss. Darmstadt

Luepke, N.P., Kemper, F.H. (1986)
The HET-CAM Test: An Alternative to the Draize Eye Test
Fd Chem. Toxic. 24, 495-496

Spielmann, H. (1994)
HET-CAM Test
From: Methods in Molecular Biology, Vol 43:
In Vitro Toxicity Testing Proto-cols Edited by: S. O'Hare and C. K. Atterwill Copyright Humana Press Inc., Totowa, NJ

van Erp, Y.H.M., Weterings P.J. (1990)
Eye Irritancy Screening for Classification of Chemicals
Toxicology in Vitro 4, 267-269

16. Appendix – 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
	07/06/2009

PRODUCT	LOT	SIZING	QUANTITY
NIOBIUM METAL	AD/4202		7.0

MARK	CUSTOMER	PACKAGING
	REACH	1/1

Element	Analysis
ppm Al	<10
ppm Be	<1
ppm C	<30
ppm Co	<1
ppm Cr	<10
ppm Cu	<1
ppm Fe	<10
ppm H	14
ppm Hf	<25
ppm Mo	<50
ppm N	23
ppm Ni	<20
ppm O	89
ppm S	<10
ppm Si	<20
ppm Ta	1267
ppm Ti	<13
ppm W	<18
ppm Zr	<7

Size Distribution

Screen (mm)	(%) Analysis

Observation

Emitted by
f/ Leandro Oliveira Lima
Chemist

Approved by
f/ Andreia Duarte Menezes Teixeira
Lab. Manager