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In vitro Cytotoxicity Assay:

Cell Growth Analysis via BCA-Staining with an Extract of Trindo-Coloring

Report

Version: Final

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BSL BIOSERVICE Study No.: 143942

Sponsor:
Trindo GmbH
Bäckerstr. 10 Rgb
81241 München
Germany

1. Contents

2. Preface

2.1. Abbreviations

ATCC	American Type Culture Collection
BCA	Bicinchoninic acid
BGBl.	Bundesgesetzblatt *(Federal Law Gazette)*
DIN	Deutsches Institut für Normung *(German Institute for Standardisation)*
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DSMZ	Deutsche Sammlung für Mikroorganismen und Zellkulturen *(German Collection for microorganism and cell culture)*
EDTA	Ethylene Diamine Tetraacetic Acid
e.g.	exempli gratia *(for example)*
EN	Europäische Norm *(European standard)*
EWG	Europäische Wirtschaftsgemeinschaft *(European Economic Community, EEC)*
FBS	Fetal Bovine Serum
G.I.	Growth Inhibition
GLP	Good Laboratory Practice
GmbH	Gesellschaft mit beschränkter Haftung *(company with limited liability)*
IEC	International Electrotechnical Commission
ISO	International Organisation for Standardisation
L929	strain L, clone 929
NCTC	National Collection of Type Cultures
Nr.	Nummer *(number)*
OECD	Organisation for Economic Cooperation and Development
QAU	Quality Assurance Unit
SOP	Standard Operating Procedures

2.2. General

Sponsor:	Trindo GmbH Bäckerstr. 10 Rgb 81241 München Germany
Study Monitor:	Mr. Felix Ewald
Test Facility:	BSL BIOSERVICE Scientific Laboratories GmbH Behringstraße 6/8 82152 Planegg Germany
BSL BIOSERVICE Study No.:	143942
Test Item:	Trindo-Coloring
Title:	*In vitro* Cytotoxicity Assay: Cell Growth Analysis via BCA-Staining with an Extract of Trindo-Coloring

2.3. Project Staff

Study Director:	Dr. Benjamin Hoy
Head of GLP Quality Assurance Unit:	Dipl.-Biol. Uwe Hamann

2.4. Schedule

Arrival of the Test Item:	18 July 2014
Start of Experiment:	24 July 2014
End of Experiment:	28 July 2014

2.5. Project Staff Signature

Study Director: Dr. Benjamin Hoy

B. Hoy

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Date: 01 Aug 2014

3. Quality Assurance

3.1. GLP Compliance

This study was performed in conformity with internal quality assurance regulations, on the basis of GLP regulations, but was not audited by the quality assurance unit. Therefore it does not have a GLP status. The test facility BSL BIOSERVICE Scientific Laboratories GmbH is certified according to the Principles of Good Laboratory Practice and accredited according to 93/42/EWG, 90/385/EWG and DIN EN ISO/IEC 17025:2005.

3.2. Guidelines

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

Biological evaluation of medical devices:

ISO 10993-1: 2009, "Evaluation and testing within a risk management process" (1)

ISO 10993-5: 2009, "Tests for *in vitro* cytotoxicity" (2)

ISO 10993-12: 2012, "Sample preparation and reference materials" (3)

3.3. Archiving

All original data generated during the conduct of the study (raw data and copy of report) are stored in the Scientific Archives of BSL BIOSERVICE Scientific Laboratories GmbH for 12 years after issue of the final report. As requested the remaining test item will be returned to the Sponsor.

4. Summary

In the present study the cytotoxic effects of Trindo-Coloring were analysed. Hereby, the test item was extracted under agitation for 24 ± 2 h with cell culture medium and the extract was incubated with L929 cells for 68 - 72 h. The protein content of the individual cultures was then analysed as a measure for cytotoxicity and compared to those of the controls.

In this study under the given conditions no leachable substances were released in cytotoxic concentrations from the test item.

5. Introduction

Cytotoxicity tests represent one of the easiest methods for the analysis of detrimental effects of substances. Cell culture techniques allow a rapid yet sensitive diagnosis of the biological reactivity of leachable or diffusable components of materials (4, 7).

The BCA test predicts cytotoxic or necrotic effects of medical devices or materials with good correlation to animal experiments and high sensitivity (5, 6).

The test item is analysed for its leachable cytotoxic contents in the BCA test. Cytotoxic effects lead to a reduction of the proliferation rate of the cells. This leads to a reduction in the protein content of the cell culture as compared to the control cultures and is detected colourimetrically after a 68 - 72 h incubation period via the BCA test (8, 9).

The BCA reagents are comprised of the water soluble and stable BCA (**Bic**inchoninic **a**cid) and an alkaline Cu^{2+} solution. The amino acids cysteine, cystine, tryptophan and tyrosine, which are a constituent of every cell, bind to these reagents, i.e these amino acids reduce Cu^{2+} to Cu^{+} which then binds to bicinchoninic acid to form a water soluble violet dye. The intensity of the dye correlates with the cell number in the culture.

5.1. Aim of the Study

This *in vitro* method analyses the cytotoxic potential of the test item. The test is carried out using the mouse cell line L929 cultured with different concentrations of an extract of the test item. The vitality of the cells or potential cytotoxic effects of the extract are registered via the protein content of the cell culture as compared to the controls.

5.2. Justification for the Selection of the Test System

L929 is a widely used and well established cell line for *in vitro* experiments since many years. It is known for its cloning efficiency and high proliferation rate.

5.3. Justification for the Selection of the Test Method

This cell culture method is applicable for the cytotoxicity analysis of all medical devices and materials which are destined for implantation or come in contact with tissue or tissue fluids for a longer period.

6. Materials and Methods

6.1. 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:	Trindo-Coloring
VAT No.:	DE 289523589
Batch No.:	1111
Expiry Date:	not applicable
Storage Conditions:	at room temperature
Type of Material:	Polyamid 12
Calculated Surface as provided by the Sponsor:	203 cm^2/test item
Sterility:	unsterile
Safety Precautions:	The routine hygienic procedures were sufficient to assure personnel health and safety.

6.2. Extraction of the Test Item

The extraction was carried out in compliance with ISO 10993-5, -12. The test item was extracted under agitation for 24 ± 2 h in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 ± 1°C. The surface/volume ratio in the assay was 3 cm^2/mL which corresponds to 100% extract concentration. After extraction the test extract was processed by sterile filtration in order to prevent a contamination of the test system. The extraction procedure did not reveal any abnormalities in the extraction medium or the test item. No changes regarding clarity, color and presence or absence of foreign material occurred in the extraction medium. The test item was tested as provided by the Sponsor.

6.3. Controls

Controls were set up in parallel to the test item cultures in order to confirm the validity of the test.

Negative control

The negative control, Polypropylene material (Greiner, Art. No. 188.271, Lot-No. E130609L), was extracted at a weight/volume ratio of 1 g/5 mL medium for 24 ± 2 h at 37 ± 1°C.

Positive control

The positive control, Dimethylsulfoxide (DMSO 99.5%, AppliChem, Lot-No. 1V004468), was set up in a final concentration of 5% in DMEM 10% FBS.

Solvent control

A solvent control, consisting of extraction vehicle (DMEM 10% FBS) alone and treated in the same way as the treatment groups, was included.

6.4. Cells

The test was carried out with L929 cells (ATCC No. CCL1, NCTC clone 929 (connective tissue mouse), clone of strain L (DSMZ)). For the test cells were cultured in 75 cm^2 or 175 cm^2 culture flasks (Greiner) in DMEM (Invitrogen) with 10% FBS-Gold (PAA Laboratories GmbH) at 37 ± 1°C and 5.0% CO_2.

6.5. Dose Groups

1. Solvent control	DMEM 10% FBS
2. Negative control	Polypropylene extracted in DMEM 10% FBS
3. Positive control	DMSO (5%) in DMEM 10% FBS
4. Test Item	6 concentrations of the test extract: 13.2%, 19.8%, 29.6%, 44.4%, 66.7% and 100%.

6.6. Experimental Procedure

The extract of the test item and the solvent control were diluted five times with DMEM 10% FBS at a ratio of 2:3. 100 µL of the different dilutions or 100 µL of the controls were given to three parallel cultures in a 96 well plate (Greiner).

Log phase L929 cultures were washed and trypsinized with Trypsin EDTA for approximately three minutes. The enzymatic reaction was stopped with DMEM 10% FBS and a single cell suspension was made at a density of 1.0 x 10^5 cells per mL. 50 µL of this cell suspension were pipetted to all cultures with the exception of the blanks. The highest concentration of the test extract in the cell cultures corresponds to a surface/volume ratio of 3 cm^2/mL. The cell culture plate was then incubated with the test extract for 68 - 72 h at 37 ± 1°C, 5.0% CO_2 / 95% air.

BCA-staining

The protein contents of the individual cultures were measured colourimetrically using the BCA reagents (Uptima). The absorption at 550 nm was measured using a micro plate auto reader.

The mean absorption (A_{550nm}) and standard deviation of the three parallel cultures was calculated and used for assessing the percentage of growth inhibition (% G.I.) following the depicted formula:

$$\% \text{ G.I.} = 100 - 100 \times \frac{(A_{550\,nm} \text{ sample}) - (A_{550\,nm} \text{ blank})}{(A_{550\,nm} \text{ control}) - (A_{550\,nm} \text{ blank})}$$

$A_{550\,nm}$ sample:	Absorption value of the test extract
$A_{550\,nm}$ blank:	Absorption value of the blank cultures (without cells)
$A_{550\,nm}$ control:	Absorption value of the solvent control

6.7. Data Analysis

According to ISO 10993-5 (3) cytotoxic effects can be based on the protein content of the cultures which is used as a measure for cell growth. Clear cytotoxicity is hereby defined as an effect leading to an inhibition of cell growth of the test extract of more than 30% as compared to the cultures treated with solvent control.

7. Results

Table 1: Results of Test Item Trindo-Coloring

	Rel. Protein content (A550) (a) 1	2	3	x	±	s	Growth inhibition in %
Blank	0.146	0.138	0.143	0.142	±	0.003	
Positive control (b)	0.238	0.248	0.246	0.244	±	0.005	88
Negative control (c)	1.052	1.108	1.093	1.084	±	0.024	0
Solvent control							
100% v/v	0.894	1.027	1.024	0.982	±	0.062	0
66.7% v/v	1.050	1.096	1.071	1.072	±	0.019	0
44.4% v/v	1.030	1.061	1.052	1.047	±	0.013	0
29.6% v/v	1.010	1.032	1.015	1.019	±	0.010	0
19.8% v/v	1.084	1.115	1.075	1.091	±	0.017	0
13.2% v/v	1.060	1.048	1.077	1.062	±	0.012	0
Test extract (d)							
100% v/v	0.870	0.850	0.855	0.858	±	0.008	15
66.7% v/v	1.030	1.000	1.017	1.016	±	0.012	6
44.4% v/v	1.056	1.080	1.067	1.068	±	0.010	0
29.6% v/v	1.009	1.021	0.986	1.005	±	0.015	2
19.8% v/v	1.055	1.077	1.071	1.068	±	0.009	2
13.2% v/v	1.021	1.053	1.068	1.047	±	0.019	2

(a) 3 parallel cultures, mean ± standard deviation

(b) 5% DMSO in DMEM 10% FBS

(c) PP material extracted in DMEM 10% FBS

(d) The test item was extracted under agitation in DMEM 10% FBS for 24 ± 2 h at 37 ± 1° C and the test extract was cultured for 68-72 h with L929 cells at a final surface/volume ratio of 3 cm^2 test item / mL culture medium.

8. Discussion and Conclusion

Changes of cell proliferation due to the presence of cytotoxic substances were analysed in a cell growth inhibition test by comparing the protein content of the cell cultures treated with an extract of the test item with that of the untreated controls.

In the present study Trindo-Coloring was extracted under agitation for 24 ± 2 h with DMEM 10% FBS. L929 cells were then incubated for 68-72 h with the following end concentrations of the test extract:

13.2%, 19.8%, 29.6%, 44.4%, 66.7% and 100%.

The highest test extract concentration corresponds to the ISO10993-5, -12 described surface/volume ratio of 3 cm^2/mL.

Growth analysis of cells cultured with the test extract showed no relevant growth inhibition of L929 cells.

The controls confirmed the validity of the study. Cell growth of the positive cultures was inhibited by 88%. The extract of the negative control did not show any inhibition of cell growth (0%).

8.1. Conclusion

In this study under the given conditions no leachable substances were released in cytotoxic concentrations from the test item.

9. Distribution of the Report

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

10. References

10.1. Guidelines

(1) ISO 10993-1: 2009, "Biological evaluation of medical devices – Part 1: Evaluation and testing within a risk management process"

(2) ISO 10993-5: 2009, "Biological evaluation of medical devices – Part 5: Tests for in vitro cytotoxicity"

(3) ISO 10993-12: 2012, "Biological evaluation of medical devices – Part 12: Sample preparation and reference materials"

10.2. Literature

(4) Guess, W.L.; Autian, J.: 1966, "Toxicity Evaluation of Lexan, Kyonar, Rilsan, Short-Term Studies", Journal of Oral Therapeutics and Pharmacology Vol 3, No.2, pp 116 – 123

(5) Autian, J. and E.O. Dillingham: 1978, "Overview of General Toxicity Testing with Emphasis on Special Tissue Culture Tests, In: In Vitro Toxicity Testing 1975-1976, in: J. Berky and C. Seherrod, Eds.",
The Franklin University Press, Philadelphia, 1978, pp 21-49

(6) Wilsnack, R.E.: 1976, "Quantitative Cell Culture Biocompatibility Testing of Medical Devices to Animal tests Biomaterials",
Medical Devices and Artificial Organs Vol. 4, pp. 235-261

(7) Stark, D.M., Shopsis C., Borenfreund, E. and Babich H.: 1986, "Progress and problems in evaluating and validating alternative assays in Toxicology",
Fd. Chem. Toxic. Vol 24, No. 6/7, pp 449-455

(8) Mosmann, T.: 1983, "Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays",
J. Immunol. Meth. 65: 5563

(9) Smith, P.K., Krohn, R.I., Hermanson, A.K., Mallia, A.K., Gartner, F.H., Provenzano M.D., Fujimoto E.K., Goeke N.M. Olson B.J. and Klenk D.C: 1985, "Measurement of Protein Using Bicinchoninic Acid.",
Analytical Biochemistry 150, 76 – 85

10.3. Internal BSL BIOSERVICE SOPs

Standard Operating Procedure (SOP), No. 9-2-1