Confidential

V0014-130

Lab No. 00T 00946 00

P.O. No. Check No. 3861

STUDY TITLE:

CYTOTOXICITY STUDY USING THE ISO ELUTION METHOD

(1X MEM Extract)

TEST ARTICLE:

Disposable Speculum & Oburator for Colonic Irrigation Equipment Model SP01/SP02

IDENTIFICATION NO.:

Batch #1 Lot #1 SP01/SP02

TEST FACILITY:

NAMSA
2261 Tracy Road
Northwood, OH 43619-1397

SPONSOR:

MARY RUTH BAKER
CLEARWATER COLON
HYDROTHERAPY INC
4451-A SOUTH PINE AVE
OCALA, FL 34480

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TABLE OF CONTENTS

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SUMMARY

An *in vitro* biocompatibility study, based on the International Organization for Standardization 10993: Biological Evaluation of Medical Devices, Part 5: Tests for Cytotoxicity: *in vitro* Methods guidelines, was conducted on the test article, Disposable Speculum & Oburator for Colonic Irrigation Equipment Model SP01/SP02, Batch #1 Lot #1 SP01/SP02, to determine the potential for cytotoxicity. A single extract of the test article was prepared using single strength Minimum Essential Medium supplemented with 5% serum and 2% antibiotics (1X MEM). This test extract was placed onto three separate confluent monolayers of L-929 mouse fibroblast cells propagated in 5% CO_2. Three separate monolayers were prepared for the reagent control, negative control and for the positive control. All monolayers were incubated at 37°C in the presence of 5% CO_2 for 48 hours. The monolayer in the test, reagent control, negative control and positive control wells was examined microscopically at 48 hours to determine any change in cell morphology.

Under the conditions of this study, the 1X MEM test extract showed no evidence of causing cell lysis or toxicity. The 1X MEM test extract met the requirements of the test since the grade was less than a grade 2 (mild reactivity). The reagent control, negative control and the positive control performed as anticipated.

Study and Supervisory Personnel: Anthony M. Jackson, BA
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Date Completed

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INTRODUCTION

The test article identified below was extracted, and the extracts were subjected to an *in vitro* cytotoxicity study for biocompatibility based on the requirements of the International Organization for Standardization 10993: Biological Evaluation of Medical Devices, Part 5: Tests for Cytotoxicity: *in vitro* Methods. The test was performed to determine whether leachables extracted from the material would cause cytotoxicity. The test article was received on January 25, 2000. The cells were first exposed to the extract on January 28, 2000, and the observations were concluded on January 30, 2000.

MATERIALS

The sample provided by the sponsor was identified and handled as follows:

Test Article:	Disposable Speculum & Oburator for Colonic Irrigation Equipment Model SP01/SP02
Identification No.:	Batch #1 Lot #1 SP01/SP02
Storage Conditions:	Room temperature
Extraction Vehicle:	Single strength Minimum Essential Medium supplemented with 5% serum and 2% antibiotics (1X MEM)
Test Article Preparation:	Based on the USP ratio of 4 g:20 ml, a 24.5 cm^2 portion of the test article was covered with 123 ml of 1X MEM. A single preparation was extracted at 37°C for 24 hours.
Negative Control Preparation:	The current NAMSA negative control material, low density polyethylene, was used as the negative control. Based on the USP ratio of 60 cm^2:20 ml, a single 30 cm^2 portion of the control material was covered with 10 ml of 1X MEM. The preparation was subjected to the extraction conditions previously described for the test article.
Reagent Control Preparation:	A single aliquot of MEM without test material was subjected to the same extraction conditions as described for the test article.
Positive Control Preparation:	The current NAMSA positive control, tin stabilized polyvinylchloride, was used to determine a cytotoxic end-point. Based on the USP ratio of 60 cm^2:20 ml, a single 60 cm^2 portion of the control material was covered with 20 ml of 1X MEM and extracted at 37°C for 24 hours. Serial dilutions were prepared (1:2, 1:4, 1:8, 1:16, 1:32) for an end-point titration.
Condition of Extracts:	Test: clear Reagent Control: clear Negative Control: clear Positive Control (undiluted): clear

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METHODS

Test System Management:

L-929, mouse fibroblast cells, (ATCC CCL 1, NCTC Clone 929, of strain L, or equivalent source) were propagated and maintained in open wells containing single strength Minimum Essential Medium supplemented with 5% serum and 2% antibiotics (1X MEM) in a gaseous environment of 5% carbon dioxide (CO_2). For this study, 10 cm^2 wells were seeded, labeled with passage number and date, and incubated at 37°C in 5% CO_2 to obtain confluent monolayers of cells prior to use. Aseptic procedures were used in the handling of the cell cultures following approved NAMSA Standard Operating Procedures.

Experimental Procedure:

Triplicate culture wells were selected which contained a confluent cell monolayer. The growth medium contained in triplicate cultures was replaced with 2 ml of the test extract. Similarly, triplicate cultures were replaced with 2 ml of the reagent control, negative control extract and the undiluted and each titer of the positive control. Each well was labeled with the corresponding lab number, replicate number, dilution (as applicable) and the dosing date. The wells were incubated at 37°C in 5% CO_2 for 48 hours.

Following incubation, the cultures were examined microscopically (100X) to evaluate cellular characteristics and percent lysis.

Evaluation Criteria:

The confluency of the monolayer was recorded as (+) if present and (-) if absent. In addition, the color of the test medium was observed and compared to the negative control medium. A color shift toward yellow was associated with an acidic pH range and a color shift toward magenta to purple was associated with an alkaline pH range. Each culture well was evaluated for percent lysis and cellular characteristics using the following criteria:

Grade	Reactivity	Observations	
0	None	Discrete intracytoplasmic granules	No lysis
1	Slight	Not more than 20% of the cells are round, loosely attached, and without intracytoplasmic granules	Not more than 20% lysis
2	Mild	Not more than 50% of the cells are round and devoid of intracytoplasmic granules	Not more than 50% lysis
3	Moderate	Not more than 70% of the cell monolayer contains rounded cells	Not more than 70% lysis
4	Severe	Nearly complete destruction of the cell monolayer	Greater than 70% lysis

For the test to be valid, the reagent control and the negative control must have had a reactivity of none (grade 0) and the positive control must have been a grade 3 or 4. The test sample met the requirements of the test if the biological response was less than or equal to grade 2 (mild). The test would have been repeated if the controls did not perform as anticipated and/or if all three test wells did not yield the same conclusion.

RESULTS

See Table I for results.

pH Observation: The test medium was similar to the negative control medium at 48 hours.

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Results and conclusions apply only to the test article tested. No further evaluation of these results is made by NAMSA. Any extrapolation of these data to other samples is the responsibility of the sponsor. All procedures were conducted in conformance with good laboratory practice and EN45001 Quality Standards (TÜV Product Services 1/96).

CONCLUSION

Under the conditions of this study, the 1X MEM test extract showed no evidence of causing cell lysis or toxicity. The 1X MEM test extract met the requirements of the test since the grade was less than a grade 2 (mild reactivity). The reagent control, negative control and the positive control performed as anticipated.

RECORD STORAGE

All raw data pertaining to this study and a copy of the final report are to be retained in designated NAMSA archive files.

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TABLE I

Well	Confluent Monolayer	Percent Rounding	Percent Cells Without Intracytoplasmic Granules	Percent Lysis	Grade	Reactivity
Test (1A)	(+)	0	0	0	0	None
Test (1B)	(+)	0	0	0	0	None
Test (1C)	(+)	0	0	0	0	None
Negative Control (1A)	(+)	0	0	0	0	None
Negative Control (1B)	(+)	0	0	0	0	None
Negative Control (1C)	(+)	0	0	0	0	None
Reagent Control (1A)	(+)	0	0	0	0	None
Reagent Control (1B)	(+)	0	0	0	0	None
Reagent Control (1C)	(+)	0	0	0	0	None
Positive Control (1A) 1:4 Dilution	(-)	80	80	80	4	Severe
Positive Control (1B) 1:4 Dilution	(-)	80	80	80	4	Severe
Positive Control (1C) 1:4 Dilution	(-)	80	80	80	4	Severe

(+) = Present (-) = Absent

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