Confidential

P.O. No. 4500268719

STUDY TITLE:

CYTOTOXICITY STUDY USING THE ISO ELUTION METHOD

TEST ARTICLE:

"ECOMASS"

IDENTIFICATION NO.:

NJ-96TP/000 NATURAL

TEST FACILITY:

NAMSA
Georgia Division

SPONSOR:

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SUMMARY

An *in vitro* biocompatibility study, based on the International Organization for Standardization (ISO 10993-5) guidelines, was conducted on the test artic1e, "ECOMASS", ID No. NJ-96TP/000 NATURAL, in order to determine the potential for cytotoxicity. Triplicate extracts of the test article were prepared using MEM. Each test extract was placed onto separate confluent monolayers of L-929 mouse fibroblast cells propagated in the presence of 5% CO_2. Separate triplicate wells were prepared for a negative control, reagent control, and positive controls. All wells were incubated at 37°C in the presence of 5% CO_2 for 48 hours. The monolayer in the test, negative, positive, and reagent control wells was examined microscopically at 48 hours to determine any change in cell morphology.

Under the conditions of this study, the test extracts showed mild evidence of causing cell lysis or toxicity. The test extracts were mildly cytotoxic and met the requirements of the test. The negative controls, reagent controls and the positive controls performed as anticipated.

Study and Supervisory Personnel: Season Weaver, BS

Approved by: Fabian R. Mejias, B.S.

Date completed: November 19, 1999

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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: Biological Evaluation of Medical Devices, Part 5: Tests for Cytotoxicity. The test was performed in order to determine whether leachables extracted from the material would cause cytotoxicity. The test article was received on 11-2-99. The cells were first exposed to the extract on 11-16-99, and the observations were concluded on 11-18-99.

MATERIALS

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

Test Article:	"ECOMASS"
Identification No.:	NJ-96TP/000 NATURAL
Storage Conditions:	Room temperature
Extraction Vehicle:	5% MEM
Test Article Preparation:	Based on the USP ratio of 4g: 20 ml, a 4g portion of the test article was covered with 20 ml of MEM. Triplicate preparations were extracted at 37°C ± 1°C for 24 hours. 2 ml of each extract was added to triplicate culture vessels and was incubated at 37°C ± 1°C in 5% CO_2 for 48 hours.
Negative Control:	The current NAMSA negative control material, low density polyethylene (Lot UP-l), was used as the negative control. Based on the USP ratio of 60 cm^2:20 ml, a 15.1 cm^2 portion of the control material was covered with 5 ml of MEM. Triplicate preparations were subjected to the same extraction conditions as described for the test article.
Reagent Control:	Triplicate aliquots of MEM without test material were subjected to the same extraction conditions as described for the test article.
Positive Control:	The current NAMSA positive control material, tin stabilized polyvinyl chloride (Lot N-1), was used to determine a cytotoxic end-point. Based on the USP ratio of 60 cm^2:20 ml, a 25.4 cm^2 portion of the control material was covered with 8.5 ml of MEM. Triplicate preparations were subjected to the same extraction conditions as described for the test article. Serial dilutions were prepared (2:2, 1:4, 1:8, 1:16, 1:32) for an end-point titration.
Condition of Extracts:	Test: Clear Negative Controls: clear, slightly alkaline Reagent Controls: clear Positive Controls: clear, slightly alkaline

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METHODS

Test System Management:

L-929 mouse fibroblast cells (ATCC CCLI, NCTC Clone 929, of strain L, or equivalent source) were propagated at 37°C in a gaseous environment of 5% carbon dioxide (CO_2) in an open flask containing Minimum Essential Medium (MEM) supplemented with 5% serum and a 2% concentration of the antibiotics penicillin, streptomycin, and amphotericin B. For this study, 10 cm^2 wells were seeded, labeled with passage number and date, and incubated at 37°C in 5% CO_2 in order 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:

A single culture well with a confluent cell monolayer was selected for each replicate of each preparation (test, negative control, reagent control, and positive control). Each well was labeled with the lab number (or appropriate control), replicate number (1, 2, or 3), and dosing date. The growth medium contained in each of three culture wells was replaced with 2 ml of the corresponding replicate of each test extract, negative control and reagent control preparation. Each replicate of the positive control was prepared by an end-point titration procedure. The culture medium in each culture well was replaced with 2ml of each positive control titer. All test and control wells were incubated at 37°C in 5% CO_2 for 48 hours.

Cell cultures were examine microscopically at 100X magnification to determine cell morphology. Observations for cytotoxicity were evaluated using the United States Pharmacopcia (USP) guidelines. Scoring for cytotoxicity was based on the following criteria:

Grade	Reactivity	Condition of Culture
0	None	Discrete intracytoplasmic granules; no cell lysis.
1	Slight	Not more than 20% of the cells are round, loosely attached, and without intracytoplasmic granules; some lysed cells are present.
2	Mild	Not more than 50% of the cells are round and devoid of intracytoplasmic granules; no extensive cell lysis and empty areas between cells.
3	Moderate	Not more than 70% of the cell monolayer contains rounded cells or is lysed.
4	Severe	Nearly complete destruction of the cell monolayer.

For the suitability of the system to be confirmed, the reagent and negative controls must have been a grade of 0 (reactivity none) and the positive controls must have been a grade of 3 or 4. The test article passes the test if all three of the monolayers exposed to the test medium showed no greater than a grade of 2 (mild reactivity). The test would have been replaced if the controls did not perform as anticipated and/or if all three test wells did not yield the same conclusion.

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RESULTS

The observations and scores obtained were as follows:

Well	Confluent Monolayer	% Cells Without Intracellular Granulation	% Rounding	% Lysis	Grade Score	Reactivity
Test (1)	(+)	(40)	(40)	(40)	2	Mild
Test (2)	(+)	(40)	(40)	(40)	2	Mild
Test (3)	(+)	(40)	(40)	(40)	2	Mild
Negative Control (1)	(+)	(0)	(0)	(0)	0	None
Negative Control (2)	(+)	(0)	(0)	(0)	0	None
Negative Control (3)	(+)	(0)	(0)	(0)	0	None
Reagent Control (1)	(+)	(0)	(0)	(0)	0	None
Reagent Control (2)	(+)	(0)	(0)	(0)	0	None
Reagent Control (3)	(+)	(0)	(0)	(0)	0	None
Positive Control (1) 1:4 Dilution	(-)	60	60	60	3	Moderate
Positive Control (2) 1:4 Dilution	(-)	60	60	60	3	Moderate
Positive Control (3) 1:4 Dilution	(-)	60	60	60	3	Moderate

(+) = Present (-) = Absent

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

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).

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CONCLUSION

Under the conditions of this study, the test extracts showed mild evidence of causing cell lysis or toxicity. The test extracts were mildly cytotoxic and met the requirements of the test. The negative controls, reagent controls, and the positive controls 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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