AR226_3295

TRADE SECRET

Study Title

H-25878: ISO Biocompatibility Testing

STUDY MONITOR: Carol Finlay, B.A.

STUDY COMPLETED ON: August 26, 2003

PERFORMING LABORATORY: North American Science Associates, Inc.
2261 Tracy Road
Northwood, Ohio 43619-1397

for

E.I. du Pont de Nemours and Company
Haskell Laboratory for Health and Environmental Sciences
Elkton Road, P.O. Box 50
Newark, Delaware 19714-0050

LABORATORY PROJECT ID: DuPont-13216

WORK REQUEST NUMBER: [redacted]

SERVICE CODE NUMBER: [redacted]

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STUDY INFORMATION

Substance Tested: [redacted]

Synonyms/Codes: • H-25878

Haskell Number: 25878

Composition: [redacted]

Known Impurities: None

Sponsor: E.I. du Pont de Nemours and Company
Wilmington, Delaware 19898
U.S.A.

Study Initiated/Completed: June 26, 2003 / (see report cover page)

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SUMMARY

Attached are reports (Appendices A - C) from North American Science Associates, Inc. The results for the H-25878 sample are:

	Test Results			
	Cytotoxicity	ISO Intracutaneous		ISO Muscle
Test Substance	(Agarose Overlay)	SC[a]	CSO[b]	Implantation (7 day)
H-25878	c	d	d	NS[e]

a Sodium chloride extract
b Cotton seed oil extract
c No evidence of cell lysis or toxicity
d No evidence of significant irritation.
Primary Irritation Index Characterization: Negligible
e Not significant

Study Monitor: Carol Finlay 26-Aug-03

Carol Finlay, B.A.
Staff Toxicologist

Date

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APPENDICES

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APPENDIX A

H-25878 Cytotoxicity Study Using the ISO Agarose Overlay Method

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Confidential [redacted]

Lab No. DuPont-13216

P.O. No. [redacted]

STUDY TITLE:

CYTOTOXICITY STUDY USING THE ISO AGAROSE OVERLAY METHOD

(SOLID)

TEST ARTICLE:

H-25878

TEST FACILITY:

NAMSA
2261 Tracy Road
Northwood, OH 43619-1397

SPONSOR:

CAROL FINLAY
DUPONT HASKELL LABORATORY
1090 ELKTON ROAD
PO BOX 50
NEWARK, DE 19714-0050

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3400 Cobb International Blvd., Kennesaw, GA 30152-7601 / 770.427.3101 / Fax 770.426.5692
9 Morgan, Irvine, CA 92618-2078 / 949.951.3110 / Fax 949.951.3260
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[redacted]

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[redacted] Lab No. DuPont-13216 [redacted]

SUMMARY

An *in vitro* biocompatibility study, based on the requirements of the International Organization for Standardization (ISO 10993-5), was conducted on the test article, H-25878, to determine the potential for cytotoxicity. An approximate 1 cm x 1 cm portion of the test article, a negative control, and a positive control were each placed on triplicate agarose surfaces directly overlaying confluent monolayers of L-929 mouse fibroblast cells. After incubating at 37°C in 5% CO_2 for 24 hours, the cell culture was examined macroscopically for cell decolorization around the test article and controls to determine the zone of cell lysis (if any). The cultures were then examined microscopically (100X) to verify any decolorized zones and to determine cell morphology in proximity to the articles.

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

Study and Supervisory Personnel:

Lionel D. Brock, BS
Jennifer N. Moritz, BA
Michelle Weingates-Furiate, BA
Lisa A. Severhof, BA

Approved by:

Martha Oswanski, BS, LAT
Technical Reviewer

July 3, 2003
Date Completed

/cal

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Ensuring Medical Device Safety and Compliance™
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INTRODUCTION

The test article identified below was 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: Test for Cytotoxicity *in vitro* Method. The test was performed to determine the potential of the test article to cause cytotoxicity. The test article was received on June 25, 2003. The cells were dosed on June 26, 2003, and the observations were concluded on June 27, 2003.

MATERIALS

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

Test Article:	H-25878
Identification No.:	Not Supplied
Storage Conditions:	Room temperature
Test Article Preparation:	[redacted]
Negative Control Preparation:	[redacted]
Positive Control Preparation:	[redacted]

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 flasks 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.

Preparation of Agarose Overlay:

The culture wells were selected which contained a confluent cell monolayer. The growth medium in each well was replaced with 2 ml of equal amounts of double strength Minimum Essential Medium supplemented with 10% serum and 4% antibiotics (2X MEM), supplemented with neutral red, and 2% agarose (final concentration 1% agarose, 1X MEM). The MEM-agarose mixture (2 ml) was then placed in the cell culture wells and allowed to solidify over the cells to form the agarose overlay.

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Experimental Procedure:

The test article was placed on the solidified agarose surface in three separate cell culture wells. Similarly, the negative control and the positive control were each placed on the solidified agarose surface in three cell culture wells. The wells were labeled with the corresponding lab number and dosing date, and incubated at 37°C in 5% CO_2 for 24 hours.

Following incubation, the cultures were examined macroscopically for cell decolorization around the test article and controls to determine the zone of cell lysis (if any). After macroscopic examination, the cell monolayers were examined microscopically (100X) to verify any decolorized zones and to determine cell morphology in proximity to the article.

Scoring for cytotoxicity was based on the following criteria:

Grade	Reactivity	Condition of Cultures
0	None	No detectable zone around or under specimen
1	Slight	Some malformed or degenerated cells under specimen
2	Mild	Zone limited to area under specimen and up to 4 mm
3	Moderate	Zone extends 5 -10 mm beyond specimen
4	Severe	Zone extends greater than 10 mm beyond specimen

NOTE: This chart (direct excerpt from USP) fails to accommodate 1 mm - 4 mm zones. The USP was notified of this. They responded that 1 mm - 4 mm zones should be categorized as mild (2).

For the suitability of the system to be confirmed, the negative control must have been a grade of 0 (reactivity none) and the positive control must have produced a zone of lysis (reactivity moderate to severe). The test article passed the test if all three monolayers exposed to the test article showed no greater than a grade of 2 (reactivity mild). The test would have been repeated if the controls did not perform as anticipated and/or if the test wells did not yield the same conclusion (e.g., one well passed and the other two wells failed).

RESULTS

The scores obtained were as follows:

ARTICLES		ZONE OF LYSIS (mm)	GRADE	REACTIVITY
Test Article:	(1)	0	0	None
	(2)	0	0	None
	(3)	0	0	None
Negative Control:	(1)	0	0	None
	(2)	0	0	None
	(3)	0	0	None
Positive Control:	(1)	6	3	Moderate
	(2)	5	3	Moderate
	(3)	5	3	Moderate

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[redacted] Lab No. DuPont-13216 [redacted]

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 ISO 17025.

CONCLUSION

Under the conditions of this study, the test article showed no evidence of causing cell lysis or toxicity. The test article met the requirements of the USP since the grade was less than a grade 2 (mild reactivity). The 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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APPENDIX B

H-25878 ISO Intracutaneous Study

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Confidential

[redacted]

DuPont-13216

Lab No. [redacted]

P.O. No. [redacted]

STUDY TITLE:

ISO INTRACUTANEOUS STUDY

EXTRACT

TEST ARTICLE:

H-25878

TEST FACILITY:

NAMSA
2261 Tracy Road
Northwood, OH 43619-1397

SPONSOR:

CAROL FINLAY
DUPONT HASKELL LABORATORY
1090 ELKTON ROAD
PO BOX 50
NEWARK, DE 19714-0050

NAMSA
Ensuring Medical Device Safety and Compliance™

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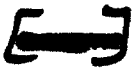

Lab No. DuPont-13216 [redacted]

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[redacted]

Lab No. DuPont-13216 [redacted]

SUMMARY

The test article, H-25878, was extracted in 0.9% sodium chloride USP solution and sesame oil, NF. These extracts were evaluated for intracutaneous reactivity based on the requirements of the International Organization for Standardization 10993: Biological Evaluation of Medical Devices, Part 10: Tests for Irritation and Sensitization.

A 0.2 ml dose of the appropriate test article extract was injected by the intracutaneous route into five separate sites on the right side of the back of each rabbit. Similarly, the corresponding reagent control was injected on the left side of the back of each rabbit. The injection sites were observed immediately after injection. Observations for erythema and edema were conducted at 24, 48, and 72 hours after injection.

Under the conditions of this study, there was no evidence of significant irritation from the extracts injected intracutaneously into rabbits. The Primary Irritation Index Characterization for the extracts was negligible.

Study and Supervisory Personnel:

Brian T. Dougan
Mark J. Menzel, RVT
Brian Brooks
Deedee M. Davis, BA
Suzanne E. Bliss
Debra S. Dunn
Martha Oswanski, BS, LAT

Approved by:

Joshua D. Moninger, BS
Technical Reviewer

July 9, 2003
Date Completed

/cal

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[redacted] Lab No. DuPont-13216[redacted]

INTRODUCTION

The test article identified below was extracted and the extracts were evaluated for biocompatibility based on the requirements of the International Organization for Standardization 10993: Biological Evaluation of Medical Devices, Part 10: Tests for Irritation and Sensitization. The purpose of the study was to determine whether leachables extracted from the material would cause local dermal irritant effects following injection into rabbit skin. The test article was received on June 25, 2003. The animals were injected on June 30, 2003, and the observations were concluded on July 3, 2003.

MATERIALS

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

Test Article:	H-25878
Identification No:	Not Supplied
Storage Conditions:	Room temperature
Vehicles:	0.9% sodium chloride USP solution (SC) sesame oil, NF (SO)
Preparation:	[redacted]

Condition of Extracts:		Test	Control
	SC:	clear with particulates	clear
	SO:	clear	clear

METHODS

Test System:

Species:	Rabbit (*Oryctolagus cuniculus*)
Breed:	New Zealand White
Source:	Myrtle's Rabbitry, Inc.
Sex:	Female
Body Weight:	2.0 kg at selection
Age:	Young adult
Acclimation Period:	Minimum 5 days
Number of Animals:	Two
Identification Method:	Ear tag

Justification of Test System:

The intracutaneous injection test in rabbits is specified in the current ISO testing standards and has been used historically to evaluate biomaterial extracts.

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9 Morgan, Irvine, CA 92618-2078 / 949.951.3110 / Fax 949.951.3280
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Animal Management:

Husbandry:	Conditions conformed to Standard Operating Procedures which are based on the "Guide for the Care and Use of Laboratory Animals."
Food:	PROLAB® High Fiber Rabbit Diet was provided daily.
Water:	Freely available, municipal (Toledo, OH) water was delivered through an automatic watering system.
Contaminants:	Reasonably expected contaminants in feed or water supplies did not have the potential to influence the outcome of this test.
Housing:	Animals were individually housed in stainless steel suspended cages identified by a card indicating the lab number, animal number, test code, sex, and date dosed.
Environmental:	The room temperature was monitored daily. The temperature range for the room was within a range of 61-72°F. The room humidity was monitored daily. The humidity range for the room was 30-77% (see DEVIATION). The light cycle was controlled using an automatic timer (12 hours light, 12 hours dark).
Facility:	NAMSA is an AAALAC International accredited facility and is registered with the United States Department of Agriculture. Additionally, NAMSA maintains an approved Animal Welfare Assurance on file with the National Institutes of Health, Office for Laboratory Animal Welfare.
Personnel:	Associates involved were appropriately qualified and trained.
Selection:	Only healthy, previously unused, thin-skinned animals free of mechanical irritation or trauma that could interfere with the test were selected.

Experimental Procedure:

Within a 4 to 18 hour period before treatment, each rabbit was weighed and clipped free of fur from the back and both sides of the spinal column to yield a sufficient injection area. The clipped area of the back was wiped with a 70% alcohol soaked gauze pad just before injection and allowed to dry. Two rabbits were prepared per pair of extracts. A 0.2 ml dose of test article extract was injected intracutaneously into five separate sites on the right side of the back of each rabbit; 0.2 ml of the reagent control was injected into five separate sites on the corresponding left side of the back. Injections were spaced approximately 2 cm apart. The appearance of each injection site was noted immediately after injection. The animals were returned to their respective cages following the procedure.

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[redacted] Lab No. DuPont-13216 [redacted]

Observations for erythema and edema were conducted at 24, 48, and 72 hours after injection. Reactions were scored on a 0 to 4 basis. Any reaction at the injection sites was also noted. The reactions were evaluated according to the following subjective rating scale:

ERYTHEMA (ER)		EDEMA (ED)	
0	No erythema	0	No edema
1	Very slight erythema (barely perceptible)	1	Very slight edema (barely perceptible)
2	Well-defined erythema	2	Well-defined edema (edges of area well-defined by definite raising)
3	Moderate erythema	3	Moderate edema (raised approximately 1 mm)
4	Severe erythema (beet redness) to eschar formation preventing grading of erythema	4	Severe edema (raised more than 1 mm, and extending beyond exposure area)

For each animal, the erythema and edema scores obtained at each time interval were added together and divided by the total number of observations. This calculation was conducted separately for each test extract and reagent control. The score for the reagent control was subtracted from the score for the test extract to obtain the Primary Irritation Score. The Primary Irritation Score of each animal was added together and divided by the total number of animals to obtain the Primary Irritation Index (PII). The Primary Irritation Index was characterized by number and description as follows: 0-0.4 (negligible), 0.5-1.9 (slight), 2.0-4.9 (moderate), 5.0-8.0 (severe). Any adverse reaction noted in the test extract was compared to the corresponding reagent control.

DEVIATION

During the course of the study, the relative humidity was found to be 30-77% in the room housing the animals. Although this lies above the range of 30-70% specified by NAMSA Standard Operating Procedure (SOP), this increase would not be expected to impact animal health and would have no impact on the conduct of the study. As a result, this temporary rise in relative humidity did not affect the integrity of the study.

RESULTS

All animals appeared clinically normal throughout the study. Results of scores for individual rabbits appear in Appendix 1. All injection sites appeared normal immediately following injection. The Primary Irritation Index (PII) and Characterization for each extract are summarized below:

Extract	Animal Number	Test Score Average	Control Score Average	Primary Irritation Score	Primary Irritation Score Total	Primary Irritation Index Characterization
SC	34742	0.0	0.0	0.0	0.0	Negligible
	34744	0.0	0.0	0.0		
SO	34742	0.5	0.3	0.2	0.2	Negligible
	34744	0.3	0.1	0.2		

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 ISO 17025.

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[redacted] Lab No. DuPont-13216 [redacted]

CONCLUSION

Under the conditions of this study, there was no evidence of significant irritation from the extracts injected intracutaneously into rabbits. The Primary Irritation Index Characterization for the extracts was negligible.

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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APPENDIX 1

ISO INTRACUTANEOUS OBSERVATIONS

Rabbit Number/ Gender	Body Weight (kg)	Extract	Scoring Interval											
			24 Hours				48 Hours				72 Hours			
			Test		Control		Test		Control		Test		Control	
			ER	ED	ER	ED	ER	ED	ER	ED	ER	ED	ER	ED
34742 Female	2.0	SC	0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
34744 Female	2.0	SC	0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
34742 Female	2.0	SO	1	0	1	0	1	0	1	0	1	0	0	0
			1	0	1	0	0	0	0	0	0	0	0	0
			1	0	1	0	1	0	0	0	1	0	0	0
			1	0	1	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	0	0	0
34744 Female	2.0	SO	0	0	0	0	0	0	0	0	1	0	0	0
			0	0	0	0	0	0	0	0	1	0	0	0
			0	0	1	0	0	0	0	0	1	0	0	0
			0	0	0	0	0	0	0	0	1	0	0	0
			0	0	0	0	0	0	0	0	1	0	0	0

ER = Erythema
ED = Edema

SC = 0.9% sodium chloride USP solution
SO = sesame oil, NF

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APPENDIX C

H-25878 USP Muscle Implantation Study

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Lab No. Du-Pont-13216

P.O. No.

STUDY TITLE:

USP MUSCLE IMPLANTATION STUDY

7 DAY

TEST ARTICLE

H-25878

TEST FACILITY:

NAMSA
2261 Tracy Road
Northwood, OH 43619-1397

SPONSOR:

CAROL FINLAY
DUPONT HASKELL LABORATORY
1090 ELKTON ROAD
PO BOX 50
NEWARK, DE 19714-0050

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3400 Cobb International Blvd., Kennesaw, GA 30152-7601 / 770.427.3101 / Fax 770.426.5592
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[redacted] Lab No. Du-Pont-13216 [redacted]

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Lab No. Du-Pont-13216 [redacted]

SUMMARY

The test article, H-25878, was implanted in muscle tissue of the rabbit. The muscle tissue was evaluated for evidence of irritation or toxicity in accordance with the guidelines of the current USP.

Negative control samples were sterilized by steam. Rabbits were implanted and were then euthanized 1 week later. Muscle tissues were excised and the implant sites were examined macroscopically.

Under the conditions of this study, the macroscopic reaction was not significant as compared to the negative control implant material. The implanted test article met the USP requirements.

Study and Supervisory Personnel:

Meridith A. Baugher, RVT, ALAT
Christine Szych, RVT
Julie Horn, RVT, LAT
Deedee M. Davis, BA
Martha Oswanski, BS, LAT

Approved by:

Joshua D. Moninger, BS
Technical Reviewer

July 16, 2003
Date Completed

/clm

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Lab No. Du-Pont-13216 [redacted]

INTRODUCTION

The test article identified below was evaluated for biocompatibility in accordance with the guidelines of the current USP. The purpose of the study was to evaluate the potential for a local irritant or toxic response to material implanted in direct contact with muscle tissue. The test article was received on June 25, 2003. The animals were implanted on July 1, 2003, and muscles were explanted on July 8, 2003.

MATERIALS

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

Test Article:	H-25878
Identification No:	Not Supplied
Storage Conditions:	Room temperature
Negative Control Article:	[redacted]
Preparation:	[redacted]

METHODS

Test System:

Species:	Rabbit (*Oryctolagus cuniculus*)
Breed:	New Zealand White
Source:	Myrtle's Rabbitry, Inc.
Sex:	Female
Body Weight Range:	3.0 kg to 3.8 kg at implantation
Age:	No particular age was prescribed for this test
Acclimation Period:	Minimum 5 days
Number of Animals:	Two
Identification Method:	Ear tag

Justification of Test System:

The rabbit is suggested as an appropriate animal model for evaluating polymer materials by the current USP testing guidelines. The muscle tissue has been used historically because the response to implanted material is easily graded and compared to a known negative control material.

Animal Management:

Husbandry:	Conditions conformed to Standard Operating Procedures which are based on the "Guide for the Care and Use of Laboratory Animals."
Food:	PROLAB® High Fiber Rabbit Diet was provided daily.

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Water:	Freely available, municipal (Toledo, OH) water was delivered through an automatic watering system.
Contaminants:	Reasonably expected contaminants in feed or water supplies did not have the potential to influence the outcome of this test.
Housing:	Animals were individually housed in stainless steel suspended cages identified by a card indicating the lab number, animal number, test code, sex, and date implanted.
Environmental:	The room temperature was monitored daily. The temperature range for the room was within a range of 61-72°F. The room humidity was monitored daily. The humidity range for the room was 30-70%. The light cycle was controlled using an automatic timer (12 hours light, 12 hours dark).
Facility:	NAMSA is an AAALAC International accredited facility and is registered with the United States Department of Agriculture. Additionally, NAMSA maintains an approved Animal Welfare Assurance on file with the National Institutes of Health, Office for Laboratory Animal Welfare.
Personnel:	Associates involved were appropriately qualified and trained.
Selection:	Healthy animals were selected. To reduce the number of animals used for testing, and to comply with the directives of the NAMSA IACUC, rabbits on this study were used previously in an unrelated test model. Any previously evaluated test or control articles did not cause a response in the animals. Complete history of animal usage is traceable in laboratory records. Animals used for previous evaluations are identified in the report.

Experimental Procedure:

Rabbits were clipped free of fur over the paravertebral muscles. An intramuscular injection of a combination of ketamine hydrochloride and xylazine general anesthetic was administered to each animal at a dose of 0.6 ml/kg. Each rabbit was then injected subcutaneously with 0.02 mg/kg buprenorphine. After the anesthetic had taken effect, the back was scrubbed with a germicidal soap, wiped with 70% isopropyl alcohol and the surgical site was painted with an antiseptic solution.

One incision was made on each side of the back through the skin and parallel to the lumbar region of the vertebral column. A stylet was placed in the hub of a loaded needle. The skin was moved to the desired location and the needle was inserted through the incision into the muscle at a 45° angle. The needle was withdrawn over the stylet, leaving the sample in the paravertebral muscle. Six test article sections were implanted in the right paravertebral muscle of each rabbit. In the opposite muscle, four negative control sections will be similarly implanted. The sections were placed at appropriately spaced intervals. The skin was closed with tissue glue. The rabbits were returned to their respective cages and monitored for recovery from the anesthetic.

Rabbits were observed daily for general health. Body weights will be recorded prior to implantation and at termination.

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At 1 week, the rabbits were euthanized by an intravenous injection of a sodium pentobarbital based drug. The paravertebral muscles were dissected free and methodically cut to locate four test article sites and two negative control sites in each rabbit. Capsule formation or other evidence of irritation was scored using low magnification and the scores were recorded as follows:

0 No capsule, no adverse reaction (other than minimal hemorrhage)
1 Up to 0.5 mm capsule or reaction area
2 0.6 to 1.0 mm capsule or reaction area
3 1.1 to 2.0 mm capsule or reaction area
4 >2.0 mm capsule or reaction area

Evaluations and Statistics:

The average macroscopic score for test implants was compared with the average score of the control sites. Calculations were rounded off to the nearest 0.1. A Reaction Index difference of 0.0 to 0.5 in scores (test minus control) was regarded as "not significant," 0.6 to 1.0 as a "trace," 1.1 to 2.0 as "slight," 2.1 to 3.0 as "moderate" and ≥3.1 as "marked." The requirements of the USP test were met if the difference between test and control score averages was not greater than 1.0. The requirements of the test were not met if the difference between the test and control score for two (or more) implant sites exceeded 1 for any animal implanted.

RESULTS

Clinical Observations: All animals appeared clinically normal throughout the duration of the study. Body weight data for individual rabbits were considered acceptable.

Macroscopic Observations: There was no visible reaction at any test or control site. This resulted in a macroscopic reaction of "not significant" tissue contact irritation. The findings for the macroscopic evaluation are shown in Appendix 1.

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 ISO 17025.

CONCLUSION

Under the conditions of this study, the macroscopic reaction was not significant as compared to the negative control implant material. The implanted test article met the USP requirements.

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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APPENDIX 1

BODY WEIGHTS AND MACROSCOPIC OBSERVATIONS

Rabbit Number/ Gender	Weight (kg)		Test	Negative Control
	Day 0	Day 7		
34097* Female	3.0	3.2	0 0 0 0	0 0
34098* Female	3.8	4.0	0 0 0 0	0 0
		Average:	0.0	0.0

*Previous use history traceable in laboratory records.

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