TEST FACILITY

NAMSA
6750 Wales Road
Northwood, OH 43619
419.666.9455

SPONSOR

Steve Dively
Applied Tungstenite Corporation
42309 Winchester Road Suites G & H
Temecula, CA 92590

STUDY TITLE

USP Intracutaneous Study in Rabbits - with Retest

TEST ARTICLE NAME

Tungstenite (WS2)

TEST ARTICLE IDENTIFICATION

Lot# 210292-40

TABLE OF CONTENTS

Page

Summary 3

1. Introduction 4
2. Materials 4
3. Test System 5
4. Animal Management 6
5. Method 6
6. Evaluation and Statistical Analysis 7
7. Results 7
8. Conclusion 7
9. Records 7
10. ISO Compliance 7
11. References 8

Appendix 1 - USP Intracutaneous Observations 9

Appendix 2 - USP Intracutaneous Observations - SO Extract Retest 10

Summary

The potential of the test article, Tungstenite (WS2), to cause irritation following intradermal injection in rabbits was evaluated based on the USP, General Chapter <88>, Biological Reactivity Tests, In Vivo. The test article was extracted in 0.9% sodium chloride USP solution (SC), sesame oil, NF (SO), alcohol in saline (AS) and polyethylene glycol (PEG). 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 of two animals. Similarly, the corresponding control was injected on the left side of the back of each animal. Observations for erythema and edema were conducted at 24, 48, and 72 hours after intracutaneous injection. A three animal retest was conducted for any extract with equivocal results.

There was no evidence of significant irritation from the SC, AS and PEG extracts injected intracutaneously into rabbits. These test article extracts met the USP requirements. Upon retest of the SO extract, there was no significant irritation. Therefore, this extract met the USP requirements.

Supervisory Personnel:

Lisa A. Severhof, B.A.
Assistant Manager, Toxicology

Colleen M. Stevenson, A.A.
Supervisor, Toxicology

Approved by:

Arizona Eugene Carter
Arizona E. Carter, B.S.
Technical Reviewer

08-13-12
Date Completed

Authorization for duplication of this report, except in whole, is reserved pending NAMSA's written approval.

1. Introduction

Purpose
The purpose of this study was to determine whether leachables extracted from the article would cause local dermal irritant effects following injection into rabbit skin.

Testing Guidelines
This study was conducted based on the United States Pharmacopeia, National Formulary, General Chapter <88>, Biological Reactivity Tests, In Vivo.

Dates

Test Article Received:	May 17, 2012
Treatment Started:	July 10, 2012
Observations Concluded:	July 13, 2012
Retest Treatment Started:	July 25, 2012
Retest Observations Concluded:	July 28, 2012

2. Materials

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

Test Article Name:	Tungstenite (WS2)
Identification:	Lot# 210292-40
Physical Description of the Test Article:	WS@ nano powder grayish-blue
Storage Conditions:	Room Temperature
Controls/Extraction Vehicles:	0.9% sodium chloride USP solution (SC)
	Sesame oil, NF (SO)
	Alcohol in saline 1:20 solution (AS)
	Polyethylene glycol 400 (PEG)
Strength, Purity, Composition or Other Characteristics:	SC: Purity: Meets requirements of USP Sodium Chloride for Injection and is certified as USP Grade. Composition: 0.9% NaCl ± 5.0% of label claim, balance is water; sodium chloride CAS #: 7647-14-5/water CAS #: 7732-18-5
	SO: Purity: Meets the requirements of National Formulary. Composition: CAS #: 8008-74-0
	AS: Composition: ethanol in saline 1:20; ethanol CAS #: 64-17-5/sodium chloride CAS #: 7647-14-5/water CAS #: 7732-18-5
	PEG: Identity: Matches infrared spectrum of polyethylene glycol 400 with average molecular weight of 380 to 420; Composition: Neat: CAS #: 25322-68-3

Extraction Procedure: The test article and the control blank (extraction vehicle without the test article) were subjected to the extraction conditions as described below. PEG test extract and control were diluted with saline to yield a 120 mg PEG/mL concentration before dosing the animal.

Vehicle	Extraction Ratio	Article Amount	Volume of Vehicle	Extraction Condition
SC	4 g:20 mL	2.0 g	10 mL	121°C for 1 hour
SO	4 g:20 mL	2.0 g	10 mL	121°C for 1 hour
AS	4 g:20 mL	2.0 g	10 mL	121°C for 1 hour
PEG	4 g:20 mL	2.0 g	10 mL	121°C for 1 hour
SO Retest	4 g:20 mL	3.0 g	15 mL	121°C for 1 hour

Condition of Extracts:

Vehicle	Treatment Group	Condition of Extract
SC	Test	Clear
	Control	Clear
SO	Test	Clear with a black tint and particulates*
	Control	Clear
AS	Test	Clear
	Control	Clear
PEG	Test	Clear with a black tint and particulates
	Control	Clear
Diluted PEG	Test	Clear
	Control	Clear
SO Retest	Test	Clear with particulates*
	Control	Clear

*An aliquot for testing was drawn off the top using a needle prior to dosing.

3. Test System

Test System

Species:	Rabbit (*Oryctolagus cuniculus*)
Breed:	New Zealand White
Source:	Robinson Services, Inc.
Sex:	Four male, three female
Body Weight Range:	No particular body weight range was prescribed for this test
Age:	Young adult
Acclimation Period:	Minimum 5 days
Number of Animals:	Seven
Identification Method:	Ear tag

Justification of Test System

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

4. Animal Management

Husbandry:	Conditions conformed to NAMSA Standard Operating Procedures that are based on the "*Guide for the Care and Use of Laboratory Animals.*"
Food:	A commercially available rabbit feed, PROLAB Hi-Fiber Rabbit – 5P25, was provided daily.
Water:	Potable water was provided *ad libitum* through species appropriate water containers or delivered through an automatic watering system.
Contaminants:	Contaminants reasonably expected in feed or water supplies were not believed to have influenced the outcome of this test.
Housing:	Animals were individually housed in stainless steel or plastic suspended cages identified by a card indicating the lab number, animal number, test code, sex, and date dosed.
Environment:	The animal housing room temperature and relative humidity were monitored daily. The temperature for the room was set to 61-72°F and the relative humidity was set to 30-70%. There were no significant temperature or relative humidity excursions that adversely affected the health of the animals. The light cycle was controlled using an automatic timer (12 hours light, 12 hours dark).
Accreditation:	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 in this study were appropriately qualified and trained.
Veterinary Care:	Standard veterinary medical care was provided in this study.
IACUC:	This procedure has been approved by the NAMSA Institutional Animal Care and Use Committee (IACUC), and is reviewed at least annually by the same committee.
Selection:	Healthy, previously unused, thin-skinned animals free of mechanical irritation or trauma that could interfere with the test were selected.

5. Method

Prior to treatment, each animal was clipped free of fur from the back and both sides of the spinal column to yield a sufficient injection area. Two animals were prepared per extract or pair of extracts. 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 animal. Similarly, the corresponding control was injected on the left side of the back of each animal. Injections were spaced approximately 2 cm apart.

The animals were returned to their respective cages following the procedure.

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:

Score	Erythema (ER)	Edema (ED)
0	No erythema	No edema
1	Very slight erythema (barely perceptible)	Very slight edema (barely perceptible)
2	Well-defined erythema	Slight edema (edges of area well defined by definite raising)
3	Moderate to severe erythema	Moderate edema (raised approximately 1 mm)
4	Severe erythema (beet redness) to slight eschar formation (injuries in depth)	Severe edema (raised more than 1 mm and extending beyond the area of exposure)

All times and temperatures reported herein are approximate and are within ranges established by the external standards described in the References section of this report and/or NAMSA standard operating procedures.

6. Evaluation and Statistical Analysis

The cumulative average erythema and edema score for each test extract and corresponding control was calculated. For each extract, a difference in average scores (test minus control) of 1.0 or less was considered to be acceptable. A difference of 0.6 to 1.0 indicated a slight, but acceptable, reaction. A difference >1.0 was considered to be unacceptable. Additionally, the average score for each test extract and control was calculated for each interval. Any adverse reaction noted in the test extract was compared to the corresponding control. Any extract considered as having equivocal results was subjected to a three animal retest.

Retest
A retest was conducted using three animals. A freshly prepared SO test extract and corresponding control were each injected and observations were conducted as in the original test. The requirements of the USP test were met if none of the retest animals showed a significant reaction to the extract.

7. Results

All animals appeared clinically normal throughout the study. Results of scores for individual animals appear in Appendix 1. Retest results for individual animals are shown in Appendix 2. The findings are summarized below:

Extract	Average Test (-) Average Control = Difference = Assessment			
SC	0.0	0.0	0.0	Passed
SO	1.3	0.0	1.3	Failed
AS	0.0	0.0	0.0	Passed
PEG	0.0	0.0	0.0	Passed
SO Retest	0.4	0.2	0.2	Passed

The difference in average scores for SC, AS and PEG was considered acceptable. The difference between the test and control average scores for the SO extract exceeded 1.0. Retesting confirmed that this extract was not an irritant.

8. Conclusion

There was no evidence of significant irritation from the SC, AS and PEG extracts injected intracutaneously into rabbits. These test article extracts met the USP requirements. Upon retest of the SO extract, there was no significant irritation. Therefore, this extract met the USP requirements.

Results and conclusions apply only to the test article tested. Any extrapolation of these data to other articles is the sponsor's responsibility.

9. Records

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

10. ISO Compliance

All procedures were certified to ISO 13485:2003.

11. References

Code of Federal Regulations (CFR), Title 9, Parts 1-4, Animal Welfare Act.

International Organization for Standardization (ISO) 13485, Medical devices - Quality management systems - Requirements for regulatory purposes (2003).

National Research Council, *Guide for the Care and Use of Laboratory Animals*, Washington, DC: National Academy Press, 2011.

Office of Laboratory Animal Welfare (OLAW), Public Health Service Policy on Humane Care and Use of Laboratory Animals.

United States Pharmacopeia 35, National Formulary 30 (USP), General Chapter <88>, Biological Reactivity Tests, In Vivo (2012).

Appendix 1 - USP Intracutaneous Observations

Extract	Animal Number	Sex	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
SC	84657	Male	0	0	0	0	0	0	0	0	0	0	0	0
SC	84659	Male	0	0	0	0	0	0	0	0	0	0	0	0
SO	84657*	Male	1	2	0	0	1	2	0	0	1	2	0	0
SO	84659*	Male	1	1	0	0	1	1	0	0	1	1	0	0
AS	84662	Male	0	0	0	0	0	0	0	0	0	0	0	0
AS	84670	Male	0	0	0	0	0	0	0	0	0	0	0	0
PEG	84662	Male	0	0	0	0	0	0	0	0	0	0	0	0
PEG	84670	Male	0	0	0	0	0	0	0	0	0	0	0	0

*All test sites immediately after dosing, and at 24, 48, and 72 hours following dosing were gray in color.

ER = Erythema
ED = Edema

Appendix 2 - USP Intracutaneous Observations - SO Extract Retest

Extract	Animal Number	Sex	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
SO	84799	Female	1	0	1	0	0	0	0	0	0	0	0	0
SO	84800	Female	1	0	0	0	0	0	0	0	0	0	0	0
SO	84801	Female	1	1	1	0	1	1	1	0	1	1	0	0

ER = Erythema
ED = Edema