

TOXIKON

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TOXIKON FINAL GLP REPORT: 11-2996-G4 AMENDED

HEMOLYSIS – RABBIT BLOOD – ASTM INDIRECT CONTACT

Test Article

SterilEnz--II/H, p/n 2H-1216-TC-5X5

Author

Sulip Goswami, M.S.

Final Report Date

August 16, 2011

Amended Report Date

August 24, 2011

COMPLIANCE

21 CFR, Part 58

Good Laboratory Practice for Non-Clinical Laboratory Studies

MANAGEMENT OF THE STUDY

Performing Laboratory

Toxikon Corporation
15 Wiggins Avenue
Bedford, MA 01730

Sponsor

PAW BioScience Products
28 Eaton Road
Eatontown, NJ 07724

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STUDY SUMMARY

The hemolytic activity of the test article, SterilEnz--II/H, p/n 2H-1216-TC-5X5, was evaluated by indirect contact using ASTM method F756-08. The test article exhibited 0.00% hemolysis above the level of hemolysis exhibited by the negative control via the indirect method. The test article is considered non-hemolytic under the experimental conditions employed.

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QUALITY ASSURANCE STATEMENT

This study was conducted in compliance with U.S. Food and Drug Administration regulations set forth in 21 CFR, Part 58.

The sections of the regulations not performed by or under the direction of Toxikon Corporation, exempt from this Good Laboratory Practice Statement, included characterization and stability of the test article and its mixture with carriers, 21 CFR, Parts 58.105 and 58.113.

The Quality Assurance Unit conducted inspections on the following dates. The findings were reported to the Study Director and to Toxikon's Management.

INSPECTIONS	DATE OF INSPECTION	DATE REPORTED STUDY DIRECTOR	DATE REPORTED MANAGEMENT
EXTRACTION	08/10/11	08/10/11	08/10/11
RAW DATA	08/16/11	08/16/11	08/16/11
FINAL REPORT	08/16/11	08/16/11	08/16/11
AMENDED REPORT	08/24/11	08/24/11	08/24/11

Michael Vandl
Michael Vandl, B.S.
Quality Assurance

8/24/11
Date

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STUDY DIRECTOR SIGNATURE AND VERIFICATION DATES

This study meets the technical requirements of the protocol. The study also meets the requirements of the Good Laboratory Practice Regulations, 21 CFR, Part 58, with the exemptions as stated in the Quality Assurance Statement.

Protocol Number: P10-3328-00A
Study Director: Sulip Goswami, M.S.
Company: Toxikon Corporation

Signature: *Sulip Goswami*

Date: 8/24/11

Study Supervisor: Sulip Goswami, M.S.

VERIFICATION DATES:

The Study Initiation Date is the date the protocol is signed by the Study Director.

Test Article Receipt: 06/21/11
Project Log Date: 07/18/11
Study Initiation Date: 08/02/11
Extraction Dates: 08/09/11-08/10/11
Technical Initiation: 08/09/11
Technical Completion: 08/10/11

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1.0 PURPOSE

The purpose of the study is to assess the hemolytic activity of a test article in indirect contact with rabbit blood.

2.0 REFERENCES

The study was based upon the following references:

- 2.1 ASTM F756–08, Standard Practice for Assessment of Hemolytic Properties of Materials, 2008.
- 2.2 ASTM F619–03, Standard Practice for Extraction of Medical Plastics, 2008.
- 2.3 Feldman, Bernard F., Joseph G. Zinkl, and Nemi C. Jain, eds. Schalm's Veterinary Hematology. 5th edition. Baltimore: Lippincott Williams & Wilkins, 2000. 858–859.
- 2.4 ISO 10993–12, 2007, Biological Evaluation of Medical Devices – Part 12: Sample Preparation and Reference Materials.
- 2.5 ISO/IEC 17025, 2005, General Requirements for the Competence of Testing and Calibration Laboratories.

3.0 COMPLIANCE

The study conformed to the current FDA 21 CFR, Part 58 – Good Laboratory Practice for Non–Clinical Laboratory Studies.

4.0 IDENTIFICATION OF TEST AND CONTROL ARTICLES

The Sponsor supplied the following information on a test requisition form or other correspondence, wherever applicable (excluding confidential or trade secret information). The Sponsor was responsible for all test article characterization data as specified in the GLP regulations.

4.1 Test Article:

Test Article Name: SterilEnz—II/H, p/n 2H-1216-TC-5X5

CAS/Code #: Not Supplied by Sponsor (N/S)

Lot/Batch #: A0211-017, Gamma Process Run 42002A

Physical State: N/S

Color: N/S

Expiration Date: N/S

Density: N/S

Stability: N/S

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Test Article: SterilEnz--II/H, p/n 2H-1216-TC-5X5

Solubility: N/S

pH: N/S

Storage Conditions: Room Temperature

Safety Precautions: Standard Toxikon Laboratory Safety Precautions

4.2 Control Articles (Toxikon Supplied):

4.2.1 Negative Control Article Name: Negative Control High Density Polyethylene (Negative Control Plastic)

Toxikon QC #: CSC-04-05-009-CC

Physical State: Solid

Color: White

Stability: Stable at Room Temperature

Storage Conditions: Room Temperature

Safety Precautions: Standard Laboratory Safety Precautions

4.2.2 Positive Control Article Name: Buna-N-Rubber

Toxikon QC #: CSC-03-07-005-CC

Physical State: Solid

Color: Black

Stability: Stable at Room Temperature

Storage Conditions: Room Temperature

Safety Precautions: Standard Laboratory Safety Precautions

4.2.3 Vehicle Control Name: Magnesium and Calcium free Phosphate Buffered Saline (PBS)

Toxikon QC #: CSC-11-05-004-CC

Physical State: Liquid

Color: Colorless

Stability: Stable at Room Temperature

Storage: Room Temperature

Safety Precautions: Standard Laboratory Safety Precautions

5.0 IDENTIFICATION OF TEST SYSTEM

The test system was citrated rabbit blood.

5.1 Animals Used in the Study:

Number and Species: Three New Zealand White rabbits (*Oryctolagus cuniculus*)

Sex: 2 males and 1 female (female was non-pregnant and nulliparous)

Weight/Age Range: 3.39–4.03 kg / at least 7 weeks old (young adult)

Health Status: healthy, previously used in other experimental procedures

Animal Purchase: Covance Laboratories, Madison, WI

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Animal Identification: ear tattoo

Acclimation: minimum 5 days, prior to the removal of a blood sample

Animal Selection: selected from larger pool and examined to ensure lack of adverse clinical signs

5.2 Animal Care and Maintenance:

Animal Room Temperature: 68 ± 5 °F

Animal Room Relative Humidity: 30–70%

Air Exchanges per Hour: a minimum of 10 changes per hour

Lights: 12-hour light/dark cycle, full spectrum fluorescent lights

Housing: individually housed

Cages: suspended stainless steel

Bedding: Alfa Cobs, Scotts Distributing, Inc., Hudson, NH (non-contact)

Animal Rations: Teklad Global High Fiber Rabbit Diet 2031, Harlan Laboratories, Madison, WI. *ad libitum*

Water: tap water, *ad libitum*

There were no known contaminants present in the feed, water, or bedding expected to interfere with the test data.

The laboratory and animal rooms were maintained as limited-access facilities.

6.0 JUSTIFICATION OF TEST SYSTEM AND ROUTE OF ADMINISTRATION

6.1 The system for the determination of hemolytic activity of a test article, when in indirect contact with rabbit blood, is recommended by ISO 10993-4 guidelines and in the ASTM Designation: F756-08, Standard Assessment of Hemolytic Properties of Materials. The guidelines have no alternative (non-animal) methods.

6.2 The test article was administered, *in vitro*, through a solvent compatible to the test system. This is the optimal route of administration available in this test system.

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7.0 EXPERIMENTAL DESIGN AND DOSAGE

7.1 Preparation of Test and Control Articles:

7.1.1 Per Sponsor request, only the fitting was tested. The pouch and the gasket were not included in the testing. The test article (149.55 cm²) was combined with 49.85 mL of PBS at a ratio of 3 cm² per 1 mL, per ISO 10993-12 and ASTM F756-08 guidelines. The test article was extracted at 70 ± 2 °C for 24 ± 2 hours.

7.1.2 The negative control (Negative Control Plastic) and positive control (Buna-N-Rubber) were extracted at a ratio of 3 cm² per 1 mL at 70 ± 2 °C for 24 ± 2 hours.

7.1.3 Properly prepared test articles were placed in separate extraction bottles and to each bottle the appropriate medium was added. The extraction medium completely covered the test article. The test and control articles were tested in triplicate.

7.1.4 Each extracting medium (control article) was prepared for parallel treatments and comparisons (blanks). Each control article was prepared in the same manner as the test article.

7.1.5 The test article appeared unchanged by the extraction procedure. It was not degraded or deformed. The extract was clear and free from particulates. Each extract was agitated vigorously prior to administration. All other test article preparation was as specified by the Sponsor.

7.2 Pre-Dose Procedure:

7.2.1 Blood Sample:

Fresh, whole rabbit blood was collected from three donors on the test day and sodium citrate was added as anticoagulant. Approximately 5 mL of blood was drawn from each animal and pooled.

7.2.2 Hemoglobin (Hb) Determination (Direct Method):

For Hb standardization, reference standards consistent with the specifications of International Committee for Standardization in Hematology (ICSH) were used. A standard curve was prepared from the stock Hb consisting of dilutions accommodating a range of 0.00 to 1.4 mg/mL. The Drabkin's reagent was used as a zero blank in the spectrophotometer and the absorbance was measured at 540 nm. The calibration curve was plotted from the values of the dilutions using mg/mL of the Hb on the y-axis and absorbances (A₅₄₀) on the x-axis. The calibration coefficient (F) is the slope of this plot. The y-intercept was approximately zero.

7.2.3 Determination of Plasma Free Hemoglobin (PFH):

A sample of pooled blood was centrifuged at 800 g for 15 minutes. A volume of 1.0 mL of plasma was added to 1.0 mL of Drabkin's reagent. Absorbance was measured at 540 nm and

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the concentration was obtained from the standard curve. The amount of PFH was calculated using the formula:

A^{PFH} = Average absorbance at 540 nm sample (1.0 mL plasma + 1.0 mL of Drabkin's reagent)

$PFH = A^{PFH} \times F \times 2$

7.2.4 Determination of Total Blood Hemoglobin Concentration:

A 20 μ L aliquot of well-mixed pooled whole blood was added to 5.0 mL of Drabkin's reagent and allowed to stand for 15 minutes. The absorbance of the solution was measured at 540 nm. The total Hb content of the blood was measured using the formula

$C = A^C \times F \times 251$. The total Hb content was adjusted to 10 ± 1 mg/mL by diluting with an appropriate amount of PBS. The Hb content was verified by using triplicate samples of 300 μ L of diluted blood to 4.5 mL of reagent (dilution factor = 16). The Hb content was measured using the formula:

$C^T = A^T \times F \times 16$

C^T = Concentration of total blood Hb

A^T = Absorbance at 540 nm of whole blood (300 μ L whole blood + 4.5 mL of Drabkin's reagent)

F = Calibration coefficient

7.3 Dose Administration:

7.3.1 A volume of 7 mL of the test article extract, negative control extract, blank, and positive control extract was placed in test tubes.

7.3.2 Addition of Blood:

Blood prepared as described in Section 7.2.4 was added to each tube at a ratio of 1 mL of blood per 7 mL of PBS.

7.3.3 The resulting solution after addition of blood was maintained at 37 ± 2 °C for 3 hours in a water bath. The tubes were gently agitated to maintain contact of the blood and material.

7.3.4 Replication:

The test article was tested in triplicate. The positive and negative controls were also tested in triplicate.

7.4 Post-Dose Procedure:

7.4.1 At the end of the incubation, the fluid was transferred into an appropriate tube and centrifuged at 800 g for 15 minutes. The supernatant was carefully collected into a screw cap vial. The test article supernatant was colorless.

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7.4.2 Determination of Supernatant Hemoglobin:

7.4.2.1 A volume of 1 mL of supernatant was added to 1 mL of Drabkin's reagent and the sample was allowed to stand for 15 minutes. The absorbance of the solution was then measured at 540 nm and Hemoglobin concentration was determined using calibration curve.

7.4.2.2 The Hemoglobin concentration in the supernatant (S) is given by:

$$S = A^S \times F \times 2$$

Where A^S = absorbance of the supernatant, F = a calibration coefficient, and 2 = the dilution factor (1 mL supernatant to 1 mL reagent).

7.4.2.3 The percentage of hemolysis or hemolysis index is calculated as follows:

$$\% \text{ hemolysis} = \frac{\text{Concentration of Hemoglobin Released in the Supernatant}}{\text{Total Hemoglobin in Tube}} \times 100$$

The absorbance of blank sample will be subtracted from the test and control articles Hemoglobin concentration.

The dilution corrected hemolysis is calculated as follows:

$$\frac{8 \times (A^S \times F \times 2) - 8 \times (A^B \times F \times 2)}{(A^T \times F \times 16) - 8 \times (A^B \times F \times 2)}$$

Where 8 is the dilution factor of 1 mL of blood and 7 mL of PBS or extract and A^S is the average absorbance of the negative, positive or the test article, A^B is the average absorbance of the blank and A^T is the average absorbance of the total hemoglobin.

This equation simplifies as:

$$\text{Dilution Corrected hemolysis} = \frac{A^S - A^B}{A^T - A^B} \times 100\%$$

8.0 EVALUATION CRITERIA

8.1 The absorbance values are used to determine the concentration of hemoglobin in the supernatant. The percent (%) hemolysis of the test article is calculated after subtraction of the blank and negative control values.

8.2 For the test to be considered valid, the percent (%) Hemolysis of the negative control needs to be in the range of 0–2%. If the negative control is above 2%, the test needs to be repeated, as per ASTM F756–08.

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8.3 If the percent (%) Hemolysis of the test article is 0–2% or less, the test article is considered non-hemolytic under the experimental conditions employed. If the percent hemolysis is between 2–5%, the test article is considered slightly hemolytic and a value higher than 5% would be concluded as hemolytic.

8.4 If the mean from the replicate test samples is < 5%, but one or more replicate sample gave a hemolytic index of > 5%, then the test is repeated with double number of test article.

8.5 If deemed necessary by the Study Director, a retest is performed using fresh blood from the same donors and a new sample of test article.

8.6 The study and its design employ methodology to minimize uncertainty of measurement and control of bias for data collection and analysis.

9.0 RESULTS

9.1 Hemoglobin Standard:

The hemoglobin concentration (C) has a coefficient of correlation with the absorbance (A) of 0.9995.

The calibration coefficient (F) is 1.563.

$$C = A \times F = A \times 1.563$$

9.2 Plasma Free Hemoglobin and Total Hemoglobin:

Sample	Absorbance Replicate 1	Absorbance Replicate 2	Absorbance Replicate 3	Absorbance Average	Standard Deviation	Concentration (mg/mL)
Plasma Free Hemoglobin (PFH)	0.1045	0.1045	0.1044	0.1045	0.0001	0.327 ^a
Total Hemoglobin (C ^T)	0.3897	0.3896	0.3896	0.3896	0.0001	9.74 ^b

a = Average Absorbance × F × 2

b = Average Absorbance × F × 16

The plasma free hemoglobin is at 0.327 mg/mL concentration that is less than the maximum of 2 mg/mL allowed by the ASTM guidelines.

The concentration of total hemoglobin used per test sample is C^T = 9.74 mg/mL.

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9.3 Hemolysis Results:

Sample	Absorbance Replicate 1	Absorbance Replicate 2	Absorbance Replicate 3	Absorbance Average	Standard Deviation	Concentration ^a (mg/mL)	Blank Subtracted Hemolysis (%)	Hemolysis Above Negative (%)
Blank (untreated)	0.0007	0.0007	0.0009	0.0008	0.0001	0.003		
Negative Control	0.0012	0.0010	0.0005	0.0009	0.0004	0.003	0.03	
Positive Control	0.0916	0.0916	0.0902	0.0911	0.0008	0.285	23.23	23.20
Test Article	0.0010	0.0005	0.0005	0.0007	0.0003	0.002	0.00*	0.00*
INDIRECT CONTACT								

a = Average Absorbance × F × 2

Blank subtracted hemolysis = $\frac{A^S - A^B}{A^T - A^B} \times 100\%$ (see Section 7.4.2.3)

* Calculated values < 0 are reported as 0.00

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10.0 CONCLUSION

The hemolytic activity of the test article, SterilEnz--II/H, p/n 2H-1216-TC-5X5, was evaluated by indirect contact using ASTM method F756-08. The test article exhibited 0.00% hemolysis above the level of hemolysis exhibited by the negative control via the indirect method. The test article is considered non-hemolytic under the experimental conditions employed.

11.0 RECORDS

- 11.1 Original raw data are archived at Toxikon Corporation.
- 11.2 A copy of the final report and any report amendments is archived at Toxikon Corporation.
- 11.3 The original final report, and a copy of any protocol amendments or deviations, is forwarded to the Sponsor.
- 11.4 All used and unused test article shall be disposed of by Toxikon, per Sponsor's request.

12.0 CONFIDENTIALITY AGREEMENT

Per corporate policy, confidentiality shall be maintained in general, and in specific accordance with any relevant agreement specifically executed between Toxikon and the Sponsor.

13.0 ANIMAL WELFARE STATEMENT

The Sponsor assured that, to the best of their knowledge, this study did not unnecessarily duplicate previous testing and that there were no non-animal alternatives acceptable for the evaluation of this test article as defined by the protocol.

No evidence of pain and distress was reported to the Veterinarian and/or Study Director.

Toxikon strictly adhered to the following standards in maintaining the animal care and use program:

United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service, 9 CFR Ch. 1 (1/1/95 edition), Subchapter A-Animal Welfare.

“Guide for the Care and Use of Laboratory Animals,” National Research Council, 2011. (NIH).

Office for Laboratory Animal Welfare (OLAW), “Public Health Service Policy on Humane Care and Use of Laboratory Animals,” Health Research Extension Act of 1985 (Public Law 99-158 November 20, 1985), Reprinted 1996.

ISO 10993-2, 2006, Biological Evaluation of Medical Devices – Part 2: Animal Welfare Requirements.

Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

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REPORT AMENDMENT PAGE

SPONSOR: PAW Bioscience
28 Eaton Road Eatontown
New Jersey, 07724

TESTING LABORATORY: Toxikon Corporation
15 Wiggins Avenue
Bedford, MA 01730

Test Article Name: SterilEnz—II/H, p/n 2H-1216-TC-5X5

CAS/Code #: Not Supplied by Sponsor (N/S)

Lot/Batch #: A0211-017, Gamma Process Run 42002A

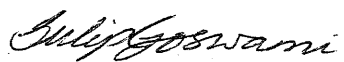
I approve of the following amendment(s) to the original report:

AMENDMENT:


Per sponsor's request the Lot Number has been changed from "A0211-017" to "A0211-017, Gamma Process Run 42002A".

I approve of the following amendment(s) to the original report:

AUTHORIZED PERSONNEL:



Sulip Goswami
Study Director



Date

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APPENDIX I Software Systems

Software	Use
Adobe Acrobat 8 Professional	Document preparation
DocuKnowledge 3.0	Lotus Domino-based document management system used for SOPs
Lotus Domino Rel. 5	Client-server application for sponsor, sample, test codes, and quotation management application databases
MS Office 2007 Small Business Suite	Business software (suite includes Word, Excel, PowerPoint, Outlook, Publisher, Office tools)
Rees CentronSQL System 2.0	Environmental monitoring and metrology system
UV_WINLAB V.2.85.04	Spectrophotometer for absorption measurement