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STUDY TITLE

ASTM Hemolysis

TEST ARTICLE NAME

Black Tecanyl MT XRO

TEST ARTICLE IDENTIFICATION

Lot: 17940

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Summary

The test article, Black Tecanyl MT XRO, Lot: 17940, was evaluated based on ASTM F756-00, Standard Practice for Assessment of Hemolytic Properties of Materials and also per the requirements of ISO 10993-4:2002, Biological Evaluation of Medical Devices - Part 4: Selection of Tests for Interactions With Blood. Blood was obtained from three rabbits, pooled, and diluted for use in this study. Diluted rabbit blood was added to triplicate tubes of the test article in calcium and magnesium-free phosphate buffered saline (CMF-PBS) and triplicate tubes of the CMF-PBS test article extract. These combinations were evaluated to determine whether direct contact with the test article or an extract of the test article would cause *in vitro* red blood cell hemolysis. Negative and positive controls were prepared in the same manner as the test article. Each tube was inverted gently to uniformly mix the contents with the blood. The tubes were then maintained for 3 hours at 37°C with periodic inversions. Following incubation, suspensions were mixed gently and centrifuged. The resulting supernatant was added to hemoglobin reagent. The absorbances of the solutions were spectrophotometrically measured at a wavelength of 540 nm.

Under the conditions of this study, the mean hemolytic index for the test article in CMF-PBS was 0%, and the mean hemolytic index for the CMF-PBS test article extract was 0%. The direct contact of the test article was nonhemolytic and the test article extract was nonhemolytic. The negative and positive controls performed as anticipated.

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1. Introduction

Purpose
The test article identified below was evaluated to determine whether the test article would cause hemolysis *in vitro* by direct contact or extraction per ASTM F756-00, Standard Practice for Assessment of Hemolytic Properties of Materials and also per the requirements of ISO 10993-4:2002, Biological Evaluation of Medical Devices - Part 4: Selection of Tests for Interactions With Blood. Hemolysis testing of medical device materials has been used historically to measure blood compatibility.

Dates
The test article was received on October 11, 2007. The test was performed on October 25, 2007.

2. Materials

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

Test Article Name: Black Tecanyl MT XRO

Test Article Identification: Lot: 17940

Storage Conditions: Room Temperature

Vehicle: Calcium and magnesium-free phosphate buffered saline (CMF-PBS)

Test Article Preparation: Based on the USP ratio of 4 g:20 ml, triplicate 2.0 g portions of the test article were covered with 10 ml of CMF-PBS to be tested as the direct contact.

For the extraction, triplicate 2.0 g portions of the test article were covered with 10 ml of CMF-PBS. These preparations were extracted with agitation at 121°C for 1 hour. Following extraction, the test extract was centrifuged at 3500 rpm for 10 minutes prior to testing.

Negative Control Preparation: High density polyethylene (HDPE) was used as the negative control.

Based on the USP ratio of 60 cm^2:20 ml, triplicate 21.0 cm^2 portions of HDPE were covered with 7.0 ml of CMF-PBS to be tested as the direct contact.

Based on the USP ratio of 60 cm^2:20 ml, triplicate 30.8 cm^2 portions of HDPE were covered with 10 ml of CMF-PBS. These preparations were subjected to the extraction conditions previously described for the test article.

Positive Control Preparation: Sterile Water for Injection (SWFI) was used for the positive control.

Triplicate 7.0 ml portions of SWFI were prepared to be tested as the direct contact.

Triplicate 10 ml portions of SWFI were subjected to the extraction conditions previously described for the test article.

Condition of Extracts: Test: clear
Negative Control: clear
Positive Control: clear

3. Test System

Test System

Species:	Rabbit (*Oryctolagus cuniculus*)
Breed:	New Zealand White
Source:	Myrtle's Rabbitry Inc.
Sex:	Male
Body Weight Range:	2.6 kg to 2.8 kg
Estimated Date of Birth:	July 16, 2007, and July 30, 2007
Acclimation Period:	Minimum 5 days
Number of Animals:	Three
Identification Method:	Ear tag

Justification of Test System

Hemolysis testing of medical device materials has historically been used to measure blood compatibility *in vitro*. Whole blood samples for use in this test were collected from the rabbits into EDTA vacuum tubes.

4. Animal Management

Husbandry:	Conditions conformed to Standard Operating Procedures that are based on the "Guide for the Care and Use of Laboratory Animals."
Food:	A commercially available rabbit feed was provided daily.
Water:	Potable water was provided *ad libitum* through species appropriate water containers or 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 animal number, sex, and blood draw date.
Environment:	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).
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 were appropriately qualified and trained.
Selection:	Only healthy animals were selected. To reduce the number of animals used for testing, and to comply with the directives of the NAMSA IACUC, one rabbit (56473) used as a blood donor for this study was used previously in an unrelated, test model. Any previously evaluated test or control articles did not cause a response in the animal. Complete history of animal usage is traceable in laboratory records. Two rabbits (56267 and 56475) had not been used previously.
Sedation, Analgesia or Anesthesia:	Sedation, analgesia or anesthesia was necessary during the routine course of this procedure.
Veterinary Care:	In the unlikely event that an animal became injured, ill, or moribund, care was conducted in accordance with current veterinary medical practice. If warranted for humane reasons, euthanasia was conducted in accordance with the current report of the American Veterinary Medical Association's Panel on Euthanasia. The objective of the study will be given due consideration in any decision and the study sponsor will be advised.

IACUC: This procedure has been approved by NAMSA Institutional Animal Care and Use Committees (IACUC), and is reviewed at least annually by the same committees. Any significant changes to this procedure were approved by the IACUC prior to conduct.

5. Preparation of Standards and Controls

Dilution Factors for Calculations
Drabkin's reagent was used as the hemoglobin reagent throughout the study. Throughout the course of the study, several dilutions of the whole blood or the blood plasma were conducted. To account for these in the calculations, the following dilution factors (DF) were used:

Plasma Hemoglobin Determination:

750 µl of plasma added to 750 µl of hemoglobin reagent

$$DF = \frac{\text{Final volume}}{\text{Volume plasma}} = \frac{1500\ \mu l\ \text{solution}}{750\ \mu l\ \text{plasma}} = 2$$

Whole Blood Hemoglobin Determination:

20 µl of whole blood added to 5 ml of hemoglobin reagent

$$DF = \frac{\text{Final volume}}{\text{Volume blood}} = \frac{5.02\ \text{ml solution}}{0.02\ \text{ml blood}} = 251$$

Diluted Blood Hemoglobin Determination:

400 µl diluted blood added to 5 ml of hemoglobin reagent

$$DF = \frac{\text{Final volume}}{\text{Volume diluted blood}} = \frac{5.4\ \text{ml solution}}{0.4\ \text{ml diluted blood}} = 13.5$$

Sample Hemoglobin Determination:

1.0 ml of Supernatant added to 1.0 ml of hemoglobin reagent

$$DF = \frac{\text{Final volume}}{\text{Volume supernatant}} = \frac{2.0\ \text{ml solution}}{1.0\ \text{ml supernatant}} = 2$$

Total Hemoglobin Concentration in each tube:

1.0 ml of diluted blood added to 7.0 ml CMF-PBS

$$DF = \frac{\text{Final volume blood/PBS - CMF}}{\text{Volume diluted blood}} = \frac{8.0\ \text{ml}}{1.0\ \text{ml}} = 8$$

Standards Preparation
The Human Hemoglobin Standard was dissolved in hemoglobin reagent. The reconstituted standard was tested at the following concentrations: 1.44, 0.800, 0.600, 0.300, 0.150, 0.0750, 0.0375, and 0.0188 mg/ml. The absorbances of the concentrations were read against a hemoglobin reagent blank in a spectrophotometer set at a wavelength of 540 nm. Using the information obtained from the absorbance readings and concentrations, a standard curve was generated.

Plasma Hemoglobin Determination
A 3 ml aliquot of the anticoagulated pooled rabbit blood was centrifuged at 700-800 Xg for 15 minutes. A 750 µl portion of the plasma (supernatant) was added to 750 µl of hemoglobin reagent. The solution was allowed to stand for 15 minutes at room temperature and the absorbance was read at 540 nm. The plasma hemoglobin concentration of the blood sample was calculated from the prepared standard curve. If the plasma hemoglobin was greater than 2 mg/ml, the blood was not used for the study.

Blood Hemoglobin Determination
Duplicate 20 µl portions of well-mixed, pooled whole blood (plasma hemoglobin ≤ 2 mg/ml) were added to 5.0 ml aliquots of hemoglobin reagent. These solutions were allowed to stand for 15 minutes at room temperature and then the absorbances were read at 540 nm. The whole blood hemoglobin concentration was calculated from the prepared standard curve.

The hemoglobin concentration of the pooled blood sample was adjusted to 10 ± 1 mg/ml by diluting with an appropriate amount of CMF-PBS. The hemoglobin concentration was confirmed by taking 400 µl of the well-mixed, diluted blood and adding it to

5.0 ml of hemoglobin reagent in triplicate. The solutions were allowed to stand at room temperature for 15 minutes and the absorbances were read at 540 nm. The diluted blood hemoglobin concentration of the sample was calculated from the prepared standard curve.

6. Method

Clot-free blood samples were collected from each rabbit (numbers 56267, 56475, 56473) into EDTA vacuum tubes on the same day as the test was performed. The blood collected from each rabbit was pooled into a borosilicate screw cap tube and mixed gently to prevent mechanical hemolysis.

The pooled rabbit blood was diluted with CMF-PBS to a total hemoglobin concentration of 10 ± 1 mg/ml. Based on a ratio of 1.0 ml diluted blood to 7.0 ml vehicle, the following tubes were prepared:

Direct Contact

1.4 ml of diluted blood and the test article in 10 ml CMF-PBS.

1.0 ml of diluted blood and the negative control in 7.0 ml CMF-PBS

1.0 ml of diluted blood and 7.0 ml of SWFI as the positive control

1.0 ml of diluted blood and 7.0 ml CMF-PBS (blank)

Extraction

1.0 ml of diluted blood and 7.0 ml of a test article CMF-PBS extract

1.0 ml of diluted blood and 7.0 ml of a negative control CMF-PBS extract

1.0 ml of diluted blood and 7.0 ml of a positive control SWFI extract

1.0 ml of diluted blood and 7.0 ml of a blank CMF-PBS extract.

The tubes were capped, inverted gently to mix the contents, and then maintained for 3 hours at 37°C with periodic inversions. Following incubation, the blood-CMF-PBS mixtures were transferred to separate disposable centrifuge tubes. These tubes were centrifuged for 15 minutes at 700-800Xg. A 1.0 ml aliquot of each test article, negative control, positive control, and blank supernatant was added to individual 1.0 ml portions of Drabkin's reagent and allowed to stand for 15 minutes at room temperature. The absorbance of each test article, negative control, positive control, and blank solution was measured at 540 nm. The hemoglobin concentration of each test article, negative control, positive control and blank solution was then calculated from the standard curve. The blank corrected percent hemolysis was calculated for each test article and the negative and positive controls as follows:

$$\text{Blank Corrected \% Hemolysis} = \frac{\text{ABS (Sample)} - \text{ABS (Blank)}}{(0.844)\,\text{ABS (Diluted Blood)} - \text{ABS (Blank)}} \times 100$$

ABS = Absorbance

7. Evaluation and Statistical Analysis

The mean blank corrected % hemolysis was calculated by averaging the blank corrected % hemolysis values determined for each of the triplicate test samples. This value is reported to the nearest 1%. The standard deviation for the replicates was also determined.

An average hemolytic index of the triplicate test samples was also calculated compared to the negative control. A hemolytic index of 2% or less was considered to be nonhemolytic. A hemolytic grade was assigned based on the following scoring scheme:

Hemolytic Index	Hemolytic Grade
0 - 2%	Nonhemolytic
2 - 5%	Slightly Hemolytic
> 5%	Hemolytic

For the suitability of the system to be confirmed, the negative control must have had a blank corrected % hemolysis value < 2% and the positive control must have had a blank corrected % hemolysis value of ≥ 8%. If either of these values were not within the acceptable range, the test was repeated with fresh rabbit blood.

8. Results

The values obtained in this study are summarized below:

TEST AND CONTROL DIRECT CONTACT SAMPLES

Sample	ABS 1	ABS 2	ABS 3	Mean Blank Corrected % Hemolysis	Standard Deviation	Mean Concentration* (mg/ml)	Hemolytic Index†
Test Article	0.003	0.003	0.004	0	0.01	0.01	0
Negative Control	0.004	0.004	0.004	0	0	0.01	
Positive Control	0.432	0.446	0.431	99.3	1.9	1.34	
Blanks	0.005	0.005	0.003	1.0*	0.3		

TEST AND CONTROL EXTRACT SAMPLES

Sample	ABS 1	ABS 2	ABS 3	Mean Blank Corrected % Hemolysis	Standard Deviation	Mean Concentration* (mg/ml)	Hemolytic Index†
Test Article	0.001	0.001	0.001	0	0	0	0
Negative Control	0.001	0.001	0.001	0	0	0	
Positive Control	0.410	0.450	0.441	98.7	4.8	1.33	
Blanks	0.001	0.001	0.001	0.2*	0		

[ ] = Not Applicable

*% Hemolysis

†Hemolytic Index calculated as follows:

Test article mean blank corrected % hemolysis – Negative control mean blank corrected % hemolysis

9. Conclusion

Under the conditions of this study, the mean hemolytic index for the test article in CMF-PBS was 0%, and the mean hemolytic index for the CMF-PBS test article extract was 0%. The test article in direct contact was nonhemolytic and the test article extract was nonhemolytic. The negative and positive controls performed as anticipated.

Results and conclusions apply only to the test article tested. Any extrapolation of these data to other samples is the sponsor's responsibility. All procedures were conducted in conformance with good manufacturing practices and certified to ISO 13485:2003.

10. Records

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

11. References

ASTM F756-00, Standard Practice for Assessment of Hemolytic Properties of Materials.

ISO 10993-4 (2002) Biological evaluation of medical devices - Part 4: Selection of tests for interactions with blood.

Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research, National Academy of Sciences (Washington: National Academy Press, 1996).

OLAW, Public Health Service Policy on Humane Care and Use of Laboratory Animals (NIH Publication).

United States Code of Federal Regulation (CFR) 9: The Animal Welfare Act.