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Toxicology Study No. 85-XC-0ENTa-11

Acute Inhalation Toxicity and Blood Absorption of 2,4-Dinitroanisole (DNAN) in Rats

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Toxicology Portfolio
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Army Institute of Public Health

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14. ABSTRACT

This toxicology study was conducted to determine the 4-hour inhalation median lethal concentration (LC50) of 2,4 -Dinitroanisole (DNAN) in male and female rats. Nose-only exposure to the highest-achievable aerosol atmosphere of DNAN (2,4 mg/L) did not induce any compound-related mortality, adverse toxic signs, body weight changes, or gross necropsy findings. A secondary objective was to determine the effect that two different routes of administration (inhalation and oral) had on the absorption of the chemical into the bloodstream. Blood samples were collected and analyzed from exposed rats at 7 time points for those exposed via inhalation and 6 different time points for those given a calculated equivalent oral dose. The results of the blood absorption study indicated that, under the stated study conditions and limitations, acute exposure to DNAN via oral gavage appears to induce higher DNAN whole blood concentrations in laboratory rats compared to those exposed via inhalation. Blood concentrations of the metabolite 2,4-Dinitrophenol (DNP) were not different between the two exposure routes.

15. SUBJECT TERMS

2,4-Dinitroanisole, DNAN, 2,4-Dinitrophenol, DNP, acute inhalation toxicity, LC50, rat, oral gavage

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Protocol No. 0ENT-24-11-07-03
Acute Inhalation Toxicity and Blood Absorption
of 2,4-Dinitroanisole (DNAN) in Rats

Data Requirement

None

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Good Laboratory Practice Compliance Statement

The study described in this report was conducted in compliance with Title 40, Code of Federal Regulations (CFR), Part 792, Good Laboratory Practice Standards, except for the following:

1. The concentrations of the test article dosing suspensions for the multi-time point blood absorption portion of the study were not verified analytically in accordance with Good Laboratory Practice Standards. The accuracy of the data reported is considered sufficient for the purposes of the acute study.

2. The statistical analyses of the multi-time point blood absorption data were conducted by the US Army Public Health Command statisticians. It is not known if these analyses were conducted in accordance with Good Laboratory Practice Standards.

ARTHUR J. O'NEILL
Study Director
Toxicity Evaluation Program

13-MAR-2015
Date

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NOVEMBER – DECEMBER 2011

1 Summary

1.1 Purpose

This study was conducted to determine the 4-hour, inhalation median lethal concentration (LC_{50}) of 2,4-Dinitroanisole (DNAN) in male and female rats. The LC_{50} is defined as the calculated atmospheric concentration of test substance expected to cause the death of 50 percent of exposed animals either on the day of exposure or within at least 14 days post exposure. In the event that no deaths occur among rats exposed to the highest-obtainable concentration, the LC_{50} is considered greater than the given concentration and no further testing is required. A secondary objective was to determine the effect that two different routes of administration (inhalation and oral) had on the absorption of the chemical into the bloodstream. Blood samples were collected from exposed rats at seven different time points for rats exposed via inhalation and at six different time points for rats exposed via oral gavage to measure the absorption of DNAN, as well as its metabolite 2,4-Dinitrophenol (DNP), into the blood and determine the degree of effect that the exposure route had on the absorption of DNAN into the blood. In addition to blood absorption, rats were also evaluated for body weight changes and clinical observations.

1.2 Conclusions

Rats were exposed nose-only to a 2.4 mg/L aerosol atmosphere of DNAN for a single 4-hour exposure. No test compound-related mortalities occurred in rats exposed during the study and no adverse toxic signs, body weight changes, or gross necropsy findings were observed in exposed rats. The LC_{50} portion of this study indicates that acute inhalation exposure to the highest-achievable concentration of DNAN aerosol (2.4 mg/L) is relatively nontoxic to rats.

The results of the multi-time point blood absorption portion indicated that, under the stated study conditions and limitations, acute exposure to DNAN via oral gavage appears to induce higher DNAN whole blood concentrations in laboratory rats compared to those exposed via inhalation. Blood concentrations of the metabolite DNP were not different between the oral and inhalation routes of exposure. Female rats appear to convert a greater proportion of DNAN to DNP than male rats when exposed via inhalation while male rats convert a greater proportion of DNAN to DNP than female rats when exposed orally.

2 References

See Appendix A for a listing of references.

3 Authority

MIPR No. MIPR1JDATHR142. This toxicology study addresses, in part, the environmental safety and occupational health requirements outlined in Army Regulations (AR) 200-1, AR 40-5, and AR 70-1; Department of Defense Instruction (DoDI) 4715.4 and Army Environmental Requirements and Technology Assessments (Department of the Army (DA), 2007a and b; DA, 2003; Department of Defense (DOD), 1996; and U.S. Army Environmental Command (USAEC), 2009). It was performed as part of an on-going effort by the U.S. Army Environmental Quality Technology (EQT), Ordnance Environmental Program Pollution Prevention Team, to produce safer ordnance. This program is under the direction of the U.S. Army Research, Development, and Engineering Command, Environmental Acquisition Logistics & Sustainment Program and EQT Pollution Prevention.

4 Background

As a result of the DOD-wide initiative to improve munitions safety, the U.S. Army is developing insensitive munitions (IM) for incorporation into its inventory of conventional ammunition and missiles. The Army's IM Program is dedicated to developing munitions that reliably perform as they are intended but are less prone to inadvertent initiation from external stimuli such as bullet/fragment impact, heat from fire, and shock from neighboring explosions (Duncan, 2002). The production of IM requires the use of intrinsically insensitive explosives that contribute to lower order responses to inadvertent external stimuli. There has been a renewed interest in DNAN's use in explosive formulations based on the lower sensitivity as a melt-cast medium observed during testing and the less stringent shipping requirements. This has led to the development of a range of melt-castable explosives at Picatinny Arsenal, collectively known as "PAX" explosives (Davies and Provatas, 2006). To support the possible fielding and full-scale production of these PAX explosives, occupational exposure guidelines need to be developed and refined using toxicity data in a mammalian system to assess any occupational health hazards associated with the use and production of this material.

DNAN is being investigated as a less sensitive direct replacement for traditional explosives such as 2,4,6-Trinitrotoluene (TNT). DNAN is a yellowish-tan powder with a wax-like consistency and is one of the components used in the formulation of an insensitive explosive referred to as IMX101 (BAE Systems, 2005). Although the use of DNAN in explosive formulations dates back to World War II, renewed interest in the energetic properties of DNAN has been fueled by the need to develop munitions that are less prone to inadvertent initiation during transport and routine handling. The reduced sensitivity to environmental stimuli and nearly equal performance during testing make DNAN-based formulations desirable replacements for currently fielded munitions (Smith and Cliff, 1999). In addition to minimizing collateral damage from weapon or ordnance accidents, insensitive munitions offer logistical advantages on the battlefield. As modern battlefields increasingly shift into populated urban centers, IM inventories represent a less desirable target for terrorists and minimize the threat to surrounding communities. Less sensitive munitions could potentially be more cost effective and efficient to transport if granted reduced DOD/Department of Transportation hazard classification rankings since DNAN is classified as a flammable solid (DA, 2008).

DNAN is moderately acutely toxic via oral administration, with an LD_{50} of 199 milligrams per kilogram (mg/kg) in the rat (Dodd and McDougal, 2002). Subchronic oral testing of DNAN revealed numerous effects occurring mostly in the highest doses of 20 and 80 mg/kg-day. Lethality was only observed in the 80 mg/kg-day dose group. Repeated oral administration of DNAN in rats also resulted in the following effects at higher doses: an apparent increase in metabolism leading to

reduced feed conversion efficiency and reduced body mass gain in male rats; changes in hematology parameters indicative of anemia, splenic enlargement, hemosiderosis, and extramedullary hematopoeisis; decreased mass of the testes and epididymides as well as degeneration and atrophy of the testicular seminiferous tubules and aspermia; and progressive development of behavioral neurotoxicity as well as associated brain lesions (U.S. Army Public Health Command (USAPHC), 2012). DNAN was reported to cause slight skin and eye irritation with reversibility in 24-48 hours, but did not cause dermal sensitization (Dodd and McDougal, 2002). Previous attempts to generate DNAN at other toxicology laboratories as a vapor/aerosol via heat vaporization yielded chamber concentrations of only 1-5 milligrams per cubic meter (mg/m^3). Acute inhalation testing of DNAN aerosolized in acetone resulted in an LC_{50} (rat, Sprague-Dawley, 4 hr) > 3 grams per cubic meter (g/m^3) (Hoffman, 2000a). One repeated-dose study was reported using DNAN aerosolized in acetone at 150, 500 and 1500 mg/m^3. All rats in the 1500 mg/m^3 and 8/10 animals in the 500 mg/m^3 group were found dead or euthanized during the exposure period. Clinical signs of toxicity observed prior to euthanasia included decreased food consumption, prostration, irregular gait, lethargy, head bobbing, poor condition, pale, backwards walking, labored breathing, and red nasal discharge. Animals exposed to 500 mg/m^3 gained less weight and consumed less feed during the first week of exposure than the acetone controls. Females in the 150 mg/m^3 group had statistically significant decreases, relative to the acetone control group, in mean hemoglobin concentrations, mean corpuscular volume, and mean corpuscular hemoglobin and increases in mean absolute monocytes and liver weight. The urine of both male and female rats exposed to 150 mg/m^3 was darker than acetone controls. The only reported compound-related microscopic finding was non-specific minimal metaplasia of laryngeal epithelium in rats exposed to 150 mg/m^3 (Hoffman, 2000b). The data from the oral subchronic study was used to calculate an occupational exposure level (OEL) for DNAN. In addition to establishing an LC_{50} for DNAN, the data from this study will also be used in an attempt to refine the proposed OEL that was previously extrapolated from oral toxicity data. Table 1 identifies the critical dates of this study.

Table 1. Critical Study Events

Critical Event	Date of Event
Animal Use Protocol Approved	July 27, 2011
Animals Received for Pilot Exposure, LC_{50} Exposure 1 & 2	November 9, 2011
LC_{50} Exposure 1	November 14, 2011
Pilot Rangefinding Blood Absorption Test	November 14,15,16,17,29, & 30, 2011
LC_{50} Exposure 2	November 16, 2011
LC_{50} Exposure 3	November 23, 2011
LC_{50} Necropsies	November 30 & December 13, 2011
Animals Received for Blood Absorption Study	December 14, 2011
Inhalation and Gavage Exposures for Blood Absorption Study	December 15 & 16, 2011
Experimental Completion	December 16, 2011
Study Completion	February 2014

5 Materials

5.1 Test Substance

Neat DNAN (CAS # 119-27-7) was produced by BAE Systems, Ordnance Systems, 4509 West Stone Drive, Kingsport, TN 37660. The certificate of analysis provided by the supplier indicated that the DNAN (lot# BAE10H281-008) was 100 percent (%) pure. The test article was dried in a vacuum oven at the testing facility at approximately 70°C for 12-48 hours to remove moisture and

re-analyzed to confirm purity. For each dosing suspension and inhalation solution, the DNAN was ground with a mortar and pestle to a fine consistency prior to use to ensure a more uniform dosing suspension and to promote melting for vapor/aerosol generation during the inhalation exposures. Oral dosing suspensions were mixed on a weight/volume basis in corn oil. Larger particles still remaining in suspension were broken up using a glass stir rod and each suspension was allowed to mix on a magnetic stir plate overnight prior to use. The dosing suspensions were also mixed continuously throughout the dosing procedure.

5.2 Animals[*†]

Each phase of this study was conducted using young adult male and female Sprague-Dawley rats obtained from Charles River Laboratories, Wilmington, Massachusetts. A total of 42 rats, 7 weeks old, were received for the acute inhalation (LC_{50}) exposures. Thirty of the 42 rats received were assigned to the LC_{50} test groups while the remaining 12 were used for the range finding blood absorption test. A total of 14 rats, 8 weeks old with a subcutaneous femoral artery catheter in place, were ordered for the multi-time point blood absorption test. Twelve of the 14 rats received were assigned to either inhalation or oral gavage test groups (one additional rat of each sex was ordered to ensure that a sufficient number of rats were available for testing in case there were clogging issues with any of the catheters following shipment). The attending veterinarian examined the animals and found them to be in acceptable health. Due to potential catheter clogging issues, rats received with femoral artery catheters did not have the standard 5-day acclimatization period, and therefore, were used for testing 1-2 days following their arrival to the testing facility. All rats were maintained in a temperature-, relative humidity-, and light-controlled room. The animal room environmental conditions were maintained at 71 ± 0.6°F and 47 ± 2.1 % relative humidity with a 12-hour light/dark cycle. A certified pesticide-free rodent chow (Harlan Teklad®, 8728C Certified Rodent Diet) and drinking quality water were available *ad libitum* except during the 4-hour exposure period. Rats were housed singly in 17-inch (length) x 9-inch (width) x 8-inch (height) solid bottom polycarbonate boxes with ALPHA-dri® bedding and suspended on a cage rack equipped with an automatic water-nipple system. Each rat was uniquely identified by number using cage cards. In addition, an animal identification number was recorded on the tail of each rat with a water-insoluble marker prior to exposure so that individual rats could be identified after exposure. (Teklad® Certified Rat Diet is a registered trademark of Harlan, Teklad. ALPHA-dri® is a registered trademark with Shepherd Specialty Papers).

5.3 Quality Assurance

The USAPHC Quality Systems Office audited critical phases of this study. Appendix B provides the dates of these audits along with the audited phase.

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[*] Research was conducted in compliance with DOD and federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council. National Academy Press, Washington, D.C. 1996.

[†] The studies reported herein were performed in animal facilities fully accredited by the American Association for the Accreditation of Laboratory Animal Care.

5.4 Study Personnel

Appendix C contains the names of persons contributing to the performance of this study.

6 Methods

6.1 General Description

This entire study protocol consisted of three separate tests utilizing two different methods of compound administration. One of the tests served as a range finding test for the more definitive multi-time point blood absorption test. The range finding blood absorption test, utilizing two additional rats per sex ordered for the each of the 3 acute inhalation LC_{50} tests (N=12), was conducted to determine if the inhalation and oral exposure levels anticipated for the multi-time point blood absorption test would allow for an accurate determination of the DNAN and/or DNP concentration in rat blood. The results of the range-finding test do not contribute meaningfully to the primary objectives of this study and, therefore, will not be reported herein.

For the acute inhalation LC_{50} tests, single groups of five male and five female rats were exposed to an atmospheric concentration of DNAN aerosolized/vaporized in air for a period of 4 hours. Rats were observed for mortality and clinical signs of toxicity during exposure and immediately following exposure. During a 14-day post-exposure recovery period, rats were observed each day for mortality and clinical signs of toxicity and weighed on selected post-exposure days. All rats were euthanized by carbon dioxide asphyxiation following the recovery period and underwent a gross pathological examination.

The multi-time point blood absorption test involved the use of two groups of three male and three female rats implanted with femoral artery catheters. One group of six rats was exposed nose-only to a DNAN aerosol/vapor for 4 hours. The second group of six rats was administered a single oral dose in corn oil via gavage at calculated equivalent doses to those exposed via inhalation. The absorption of DNAN into the bloodstream of the rats exposed via inhalation and its conversion to DNP was determined from blood samples collected from each surviving rat at seven nominal time points during each exposure: (1) approximately 1-2 hours prior to the exposure (2) approximately 1 hour after the initiation of the exposure period, (3) approximately 2 hours after the initiation of the exposure period, (4) at the conclusion of the 4-hour exposure period, (5) approximately 4 hours after the conclusion of the exposure period, (6) approximately 8 hours after the conclusion of the exposure period, and (7) following an overnight recovery period. The absorption of DNAN into the bloodstream of the rats orally gavaged and its conversion to DNP was determined from blood samples collected from each surviving rat at six nominal time points during the dosing period: (1) approximately 1-2 hours prior to dosing (2) approximately 1 hour after dosing, (3) approximately 2 hours after dosing, (4) approximately 4 hours after dosing, (5) approximately 8 hours after dosing, and (6) following an overnight recovery period. In addition to blood analysis, rats were also placed in metabolism cages during overnight recovery for collection of urine samples. Clinical observations were collected on all rats throughout the day of exposure/dosing and prior to euthanasia following the collection of the final blood sample.

The experimental design and general procedures related to the exposure chamber generation and the oral gavage procedures of this study were conducted under the USAPHC Portfolio of Toxicology Standing Operating Procedure for conducting acute inhalation and acute oral toxicity studies (USAPHC, 2010a and b). These SOPs are modeled on the U.S. Environmental Protection Agency (EPA) Office of Chemical Safety and Pollution Prevention (OCSPP) Health Effects Test

Guidelines, OCSPP 870.1300, Acute Inhalation Toxicity and OCSPP 870.1100, Acute Oral Toxicity (EPA, 1998 and 2002).

6.2 Inhalation Exposures for Acute LC_{50} Test and Inhalation Portion of Multi-Time Point Blood Absorption Test

6.2.1 Selection of Exposure Chamber Design Concentration

The design concentration of the DNAN condensation aerosol atmosphere in the exposure chamber was selected based on the highest obtainable concentration achieved (up to a limit concentration of 2 mg/L) during method development work. Multiple attempts were made during method development to generate a limit concentration of 2 mg/L however, due to the limited vaporization of DNAN as well as the inherent inefficiency of condensation aerosol atmospheres, the highest reproducible DNAN atmosphere concentration obtained was approximately 1 mg/L. The DNAN could not be generated as a dry particulate atmosphere because of its wax-like consistency and inability to be fed through a dry-material feeder. The DNAN vapor/aerosol atmosphere was also favorable because it accurately simulated the conditions possibly experienced by munitions workers at production facilities. Therefore, the design concentration for the first LC_{50} exposure was selected to be approximately 1 mg/L. It was discovered after the second LC_{50} exposure that the chamber concentration was more sensitive to adjustments in carrier gas flow than beaker mantle temperature adjustments which allowed exposure concentrations to exceed 2 mg/L.

6.2.2 Exposure System

6.2.2.1 Test Atmosphere Generation

Chamber atmospheres of DNAN aerosol were generated dynamically in air within the exposure chamber. Test atmospheres were generated by heating powdered DNAN in a fabricated 3-neck glass generation vessel to approximately 270°C with a 600-milliliter (mL) beaker heating mantle. The resulting DNAN condensation aerosol atmosphere was swept through glass tubing using a stream of nitrogen gas at flow rates ranging from 0.6 to 1.4 liters/minute. The glass tubing and top of the generation vessel was wrapped with heat tapes individually controlled by variacs to minimize DNAN condensation prior to entry into the exposure chamber. Filtered houseline air was then introduced via a port on the top of the exposure chamber to provide adequate test atmosphere mixing in the chamber and to maintain oxygen levels above 19 percent. Chamber concentrations were adjusted through changes in both beaker mantle temperature adjustments and nitrogen flow rates. Test atmospheres were exhausted through an impinger containing acetone to scrub the DNAN using a Radeco model AVS-28A vacuum pump prior to discharge into a fume hood. The rate of exhaust from the exposure chamber was sufficient to minimize leakage of the test atmosphere from the chamber (see Figure 1).

6.2.2.2 Exposure Chamber

The exposure chamber was a glass cylinder (12-inch height x 16-inch diameter x ¼-inch walls) with a nominal internal volume of approximately 36 liters. The open end of the exposure chamber was fitted with a polymethyl-methacrylate faceplate. The faceplate functioned to seal the opening of the exposure chamber and to support the nose-only restrainers during the exposure.

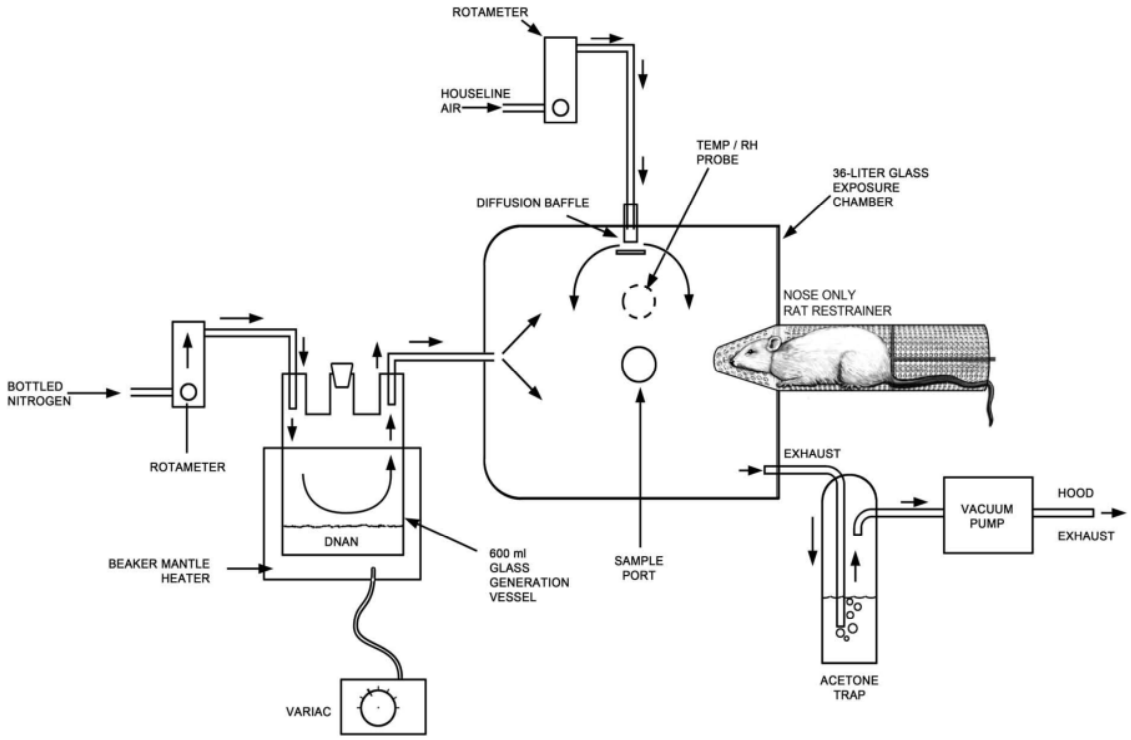

Figure 1. Generation/Exhaust/Exposure System

6.2.2.3 Exposure Mode

Animals were exposed nose-only to condensation aerosol atmospheres of DNAN such that only their heads were positioned within the exposure chamber and the bodies of these rats were positioned outside the exposure chamber. Rats were individually contained during each exposure in perforated, stainless steel cylinders with conical nose pieces. Rats were positioned in the exposure cylinder such that their noses were at the conical end of the cylinder. In order to secure the rat in this position, a plastic disk with a hole in the center was inserted over the tail of each rat and positioned within the cylinder close to the base of the rat's tail so as to prevent the rat from backing out of the rear of the cylinder. Each exposure cylinder was inserted into one of the holes in the faceplate of the exposure chamber such that the head of each rat extended into the exposure chamber (nose-only).

6.2.2.4 Exposure Duration

With the exception of the first acute LC_{50} exposure (total of three exposures) the animals for the remainder of the acute LC_{50} exposures and the inhalation portion of the multi-time point blood absorption test were exposed for 4 hours to the test atmosphere. Due to problems with the generation system, the first LC_{50} exposure was terminated approximately 2 hours early. Although these animals were retained for the 14-day recovery period and received a gross necropsy, the

results of the exposure will not be reported herein. In order to accommodate the blood collection schedule for animals in the multi-time point blood absorption test, these rats were loaded into the exposure system chamber in staggered increments. The time that each rat was loaded into the exposure system was recorded in the study records and represented the beginning of the exposure period for that rat. These rats were then removed from the exposure chamber for 6-9 minute periods for the 1- and 2-hour bleed time points. The total time that the rats were not in the exposure chamber during the 1-and 2-hour bleed time points was made up at the end of the exposure to ensure that each rat received a 4-hour exposure.

6.2.3 Characterization of Exposure Chamber Atmosphere

6.2.3.1 Test Substance Atmospheric Concentration

During method development, various sampling techniques were used to adequately characterize the chamber atmosphere to determine if a vapor component was present. Numerous tube samples were collected in line with the gravimetric sampling train and analyzed by gas chromatography (GC). The results of the GC analysis determined that no significant vapor component was present in the chamber. Additional tube samples were collected during the animal exposures to confirm that no vapor component was present. Based on data collected during the method development phase, it was verified that gravimetric analysis was the most accurate method to characterize the condensation aerosol present in the chamber during the animal exposures.

The atmospheric concentration of the DNAN condensation aerosol was determined at regular intervals (e.g., 30-minutes) during all inhalation exposures. A total of 10 to 17 samples were collected from the exposure chamber during each of the exposures. Known volumes of chamber atmosphere were drawn from a sampling port in the side of the exposure chamber representative of the animals' breathing zone. Samples were drawn through a 25-millimeter filter cassette that contained a dried, pre-weighed Gelman glass fiber (Type A/E) filter followed by an Ovs, Xad-2 tube (SKC Inc.). The purpose of the Xad-2 tube was to trap the remaining vapor component of the DNAN chamber atmosphere. All filters were weighed on a Cahn model C-34 Microbalance. The atmospheric concentration of the DNAN condensation aerosol was calculated from the difference in the pre- and post-sampling filter weights divided by the volume of chamber atmosphere sampled. Three of the Xad-2 tubes and glass fiber filters per inhalation exposure were submitted to the Army Institute of Public Health (AIPH) Laboratory Sciences (LS) Portfolio for extraction and analysis. Each glass fiber filter and Xad-2 tube was extracted and diluted with isoamyl acetate to fit within the calibration curve and analyzed using an Agilent 6890 gas chromatograph equipped with an electron capture detector.

6.2.3.2 Particle Size Analysis

Two samples to determine atmospheric particle size distribution (mass median aerodynamic diameter) of the DNAN condensation aerosol were collected during the inhalation exposures. One sample was collected during the first hour of the exposure and the second sample was collected during the final hour of the exposure. All particle size samples were collected using a Sierra® Series 210 8-Stage Cascade Impactor fitted with a Cyclone Preseparator and Anderson model SE113 Constant Flow Air Sampler. Particle size sample data were analyzed by log normal regression of particle size versus cumulative relative mass (USAPHC, 2011). (Sierra® is a trademark of Sierra Instruments Inc.).

6.2.3.3 Environmental Monitoring

Chamber airflow was set at the beginning of the exposure to achieve at least 10 air changes per hour. The airflow fed into the top of the exposure chamber was monitored continually with a Fischer & Porter model 10A3135N flowmeter. Generation vessel nitrogen flow was monitored continually with a Dwyer 5 liter/minute flowmeter. Chamber temperature was targeted at 22 ± 2 ºC and relative humidity was targeted between 30 and 70 percent. Chamber temperature and humidity were measured with an Omega model RH411 digital thermo-hygrometer thermometer. Oxygen was monitored using a Teledyne GB300 portable oxygen analyzer. Airflow, temperature, and relative humidity readings were recorded multiple times during each exposure.

6.3 Oral Gavage Portion of Multi-Time Point Blood Absorption Test

6.3.1 Calculation of Equivalent Oral Dose

The purpose of the multi-time point blood absorption test was to compare the absorption of DNAN into the blood of rats exposed via inhalation to that of rats given an equivalent oral dose. In addition the concentration of DNP, a known metabolite of DNAN, was also monitored in the blood of the exposed rats. On the day following the conclusion of the inhalation portion of the test, an average chamber concentration was calculated for the 4-hour exposure using the gravimetric filter samples collected during the exposure. The following formula was employed to estimate an equivalent oral dose for the animals to be gavaged (Rusch, 2009):

$$\text{Oral Dose (mg/kg)} = \frac{\alpha * \text{exposure level} * \text{minute volume} * \text{exposure length}}{\text{body weight}}$$

where:

α	= amount retained in respiratory system (assume 0.9 or 90 percent in the absence of additional information)
exposure level	= average concentration of inhalation exposure (mg/m^3)
minute volume	= amount of air inhaled per minute (approx. 0.00016 m^3/min for rats)
exposure length	= total minutes of inhalation exposure
body weight	= animal weight (kg)

All calculations were performed using body weights obtained within one hour of initiating the dosing procedure.

6.3.2 Administration of Test Substance

The animals were not fasted prior to dosing since the inhalation animals were not fasted prior to exposure. The dosing procedure for each animal was staggered by a period of approximately 4 minutes to accommodate the blood sampling schedule and all dosing was performed using a 16 gauge x 2-inch stainless steel gavage needle. A 16 milligram per milliliter (mg/mL) suspension of DNAN in corn oil was used for oral gavage and dosage volumes ranged from 7.0 – 9.8 mL of suspension per kg bodyweight.

6.4 Blood/Urine Sample Analysis During Multi-Time Point Blood Absorption Test

6.4.1 Collection of Blood Samples

The concentration of DNAN absorbed into the bloodstream, as well as its conversion to DNP, of exposed rats via nose-only inhalation or oral gavage was determined from blood samples collected from each surviving rat at seven selected time points for the inhalation portion and six selected time points for the oral gavage portion. All blood samples were preserved at the time of collection by immediately injecting the 100 microliter aliquot of the blood sample into 1 mL of water. Samples were tightly capped and refrigerated at approximately 4°C immediately after collection until the time of delivery for analysis.

6.4.2 Collection of Urine Samples

Surviving animals exposed via inhalation or oral gavage were placed in metabolism cages following the 8-hour post-exposure blood sample on the day of exposure for overnight urine collection. Each animal was in the metabolism cage for a period of approximately 15-16 hours. The following day the animals were removed from the metabolism cages for the final blood sample and the urine samples were transferred to Becton-Dickenson Falcon™ 15-mL conical centrifuge tubes. Each sample was centrifuged for approximately 30 minutes at slow speed to remove any particulate contamination. The liquid urine samples were then transferred to new centrifuge tubes and refrigerated at approximately 4°C until the time of delivery for analysis. (Falcon™ is a trademark of Becton, Dickinson and Co.).

6.4.3 Analysis of Blood/Urine Samples

The AIPH LS personnel analyzed the concentration of DNAN and DNP in the blood and urine samples. Prior to LS analysis, 1 mL of each urine sample was extracted with isoamyl acetate and centrifuged to separate the organic layer from the water. The blood samples were centrifuged to separate the blood cells from the collection medium. The blood samples were extracted in the same manner as the urine samples and both were diluted as necessary to bring the sample concentrations within the range of instrument calibration. All blood/urine samples were analyzed using an Agilent 6890 Gas Chromatograph/Electron Capture Detector with a J&W DB-17 analytical column. A J&W DB-1 analytical column was used for confirmation.

6.5 Body Weights and Clinical Observations

All rats were weighed and observed prior to all exposures. The animals used for the acute LC_{50} tests were weighed and observed on the day of exposure, the day following exposure, several times during the 14-day observation period, and just prior to euthanasia. The animals used for the multi-time point blood absorption test were weighed and observed on the day of exposure and on the day following exposure prior to euthanasia.

6.6 Pathology

All rats exposed for the acute LC_{50} tests were euthanized by carbon dioxide asphyxiation following the 14-day observation period and underwent a gross pathological examination. Surviving animals used for the multi-time point blood absorption test were euthanized by sodium pentobarbital

injection into the femoral artery catheter following the final blood sample and were not submitted for necropsy.

6.7 Statistical Analysis of Data

For the two acute LC_{50} toxicity exposures, the test substance generated at the highest-obtainable concentration did not result in any test substance mortality. Therefore, the LC_{50} for DNAN generated as described above is reported as greater than the highest-obtainable concentration. For the multi-time point blood absorption test, a comparison of the concentration of DNAN and DNP in the blood samples across times was performed with an analysis of variance (ANOVA) on the area-under-curve (AUC) for each rat. The AUC for each rat was calculated using the rectangle method. An ANOVA was then performed on the 4-hour AUC values with both gender and route of administration as factors. The number of pre-term deaths in those animals orally gavaged with DNAN prevented the analysis of data obtained after 4 hours post-exposure. A repeated-measures ANOVA was used to compare mean 4-hour DNAN and DNP AUC's by gender and route of administration. Statistical significance for all tests was defined as $p<.05$. Descriptive statistics (e.g., mean, standard deviation, and standard error of the mean) were used to summarize experimental data (e.g., atmospheric concentrations) as well as DNAN and DNP blood concentrations post 4-hours because pre-term deaths prevented statistical analysis.

7 Results

7.1 Generation Method Development

Prior to the initiation of the inhalation test exposures, pre-test trials were conducted to determine the most suitable method of generating test atmospheres of DNAN. The initial goal of this preliminary work was to determine if a reasonably stable atmospheric concentration of approximately 2 mg/L DNAN could be achieved. The generation system used for the animal exposures was selected based on the flammable nature of DNAN, the ability to generate test atmospheres with solid DNAN, and the ability to generate stable atmospheres of DNAN at the highest-achievable concentration and acceptable particle size. The same generation system was used for both inhalation phases of the study.

7.2 Chamber Distribution of Test Atmosphere

Prior to initiation of the inhalation test exposures, a study of the chamber distribution of the DNAN condensation aerosol concentration was performed (USAPHC, 2010c). No significant differences were detected between gravimetric samples taken from the sample port on the side of the chamber and those obtained from the chamber faceplate during method development exposures. The distribution of the DNAN condensation aerosol atmosphere at various locations in the chamber faceplate was also considered uniform when the top and bottom rows of exposure ports were excluded. Distribution of atmospheric DNAN concentrations in the exposure chamber data are presented in Appendix D.

7.3 Exposure Chamber Concentration and Conditions During Animal Exposure

7.3.1 Atmospheric Concentration of Test Chemical

Two inhalation exposures were conducted for the acute LC_{50} portion of the study and one inhalation exposure was conducted for the multi-time point blood absorption test. The mean atmospheric concentration of the DNAN condensation aerosol in the exposure chamber during the two acute LC_{50} tests were determined to be 0.73 ± 0.10 mg/L and 2.4 ± 0.25 mg/L. The mean atmospheric concentration of the DNAN condensation aerosol in the exposure chamber during the multi-time point blood absorption exposure was determined to be 0.86 ± 0.15 mg/L. Analysis of the 3 Xad-2 tubes per LC_{50} exposure revealed negligible DNAN vapor concentrations in the exposure chamber and, therefore, only the aerosol component was used for determination of the chamber concentration. Results of the extraction and analysis of three glass fiber filters per LC_{50} exposure were considered to be consistent with the gravimetric concentrations. Analytical concentrations ranged from 79 to 101 percent of the gravimetric concentrations for both LC_{50} exposures. One additional LC_{50} exposure was conducted but was aborted early due to problems with the generation system. The results from this shortened exposure are not included in this report. Exposure concentration data are presented in Appendix E and summarized in Table 2.

Table 2. Summary of DNAN Exposure Chamber Concentrations

Exposure	Gravimetric Atmospheric Concentrations of DNAN (mg/L)			
	Mean	S.D.	Range	N
LC_{50} Exposure 1	0.7261	0.10493	0.562-0.844	11
LC_{50} Exposure 2	2.3881	0.24521	1.843-2.690	10
Multi-Time Point Blood Absorption	0.8561	0.15350	0.622-1.086	15

Legend:
mg/L = milligrams per liter
S.D. = standard deviation
N = number of samples collected
Note:
Values reported to 3 significant figures

7.3.2 Nominal Concentration of Test Substance

The nominal concentration is the theoretical atmospheric concentration calculated when the total volume of test substance delivered to the generation system is divided by the total airflow of the generation system. A nominal concentration for the first LC_{50} exposure was not calculated due to method development work performed post-exposure that prevented the determination of a final test material weight. The nominal concentration of the total DNAN test atmosphere for the second LC_{50} exposure was calculated to be 21.2 mg/L ([64 g x 10^3 (mg/g)] ÷ 3021 total liters of nitrogen and air). The nominal concentration of the total DNAN test atmosphere for the multi-time point blood absorption exposure was calculated to be 4.8 mg/L ([17g x 10^3 (mg/g)] ÷ 3532 total liters of nitrogen and air.

7.3.3 Particle Size of Test Substance

The particle size of the test atmosphere, characterized by measurement of the Mass Median Aerodynamic Diameter (MMAD), was determined twice during the conduct of each of the inhalation exposures. The MMAD of the first LC_{50} exposure atmosphere ranged from 7.4 to 8.8 microns (µm), with 0 percent of the particles less than 1 µm, 8-16 percent of the particles less than 4 µm, and 60-70 percent of the particles less than 10 µm. The MMAD of the second LC_{50} exposure atmosphere ranged from 5.6 to 5.8 µm, with 0 percent of the particles less than 1 µm, 13-17 percent of the particles less than 4 µm, and 94 percent of the particles less than 10 µm. The MMAD of the test atmosphere for the inhalation portion of the multi-time point blood absorption study ranged from 4.6 to 4.9 µm, with 0 percent of the particles less than 1 µm, 26-30 percent of the particles less than 4 µm, and 98-99 percent of the particles less than 10 µm. The desired MMAD for inhalation toxicology studies typically ranges from 1-4 µm, however, the MMAD for this study was higher than desired during each of the exposures. The reason for the increased particle size was due to the method of generation, which was a direct result of the physical properties of the test material. The test material was a wax-like solid that could not be dispersed in the air as a dry powder. As discussed in Section 6.2.2.1, the test material was heated and generated as a condensation aerosol, which actually represented a realistic exposure scenario for individuals involved with this material in a munition plant. Condensation aerosols make it difficult to control the particle size of the test atmosphere due to various factors (e.g., sublimation, condensate nuclei, transit time of the atmospheric particles). The particle sizes generated during this study were the result of attempts to deliver the most reasonable MMAD based on physical constraints of the test material, and therefore, were considered to be acceptable for assessing the inhalation toxicity of this test material. Particle size data is summarized in Table 3.

Table 3. Summary of DNAN Exposure Particle Sizes

Exposure	Mass Median Aerodynamic Diameter (µm)	Geometric Standard Deviation	% Particles by Mass		
			<1 µm	<4 µm	<10 µm
LC_{50} Exposure 1*	7.4 – 8.8	1.8 – 1.8	0.0	8 - 16	60 - 70
LC_{50} Exposure 2*	5.6 – 5.8	1.3 – 1.4	0.0	13 - 17	94 - 94
Multi-Time Point Blood Absorption*	4.6 – 4.9	1.4 – 1.4	0.0	26 - 30	98 - 99

Legend:
µm = micrometer
% = percent
Note: *Two particle size samples collected during exposure.

7.3.4 Exposure Chamber Environmental Conditions

Chamber environmental conditions were similar between the LC_{50} and multi-time point blood absorption exposures. Airflow to the exposure chamber during all of the exposures was maintained at 10 L/min. Chamber temperatures for the exposures ranged from 21°C to 25°C and were slightly outside of the targeted range of 22 ± 2°C during certain periods of the exposures. Chamber relative humidity for the exposures ranged from 15 to 33 percent and was slightly lower than the targeted range of 30 to 70 percent due to the heat required to generate DNAN. Oxygen levels remained above 19% throughout all exposures with the exception of the first reading during the second LC_{50} exposure. Due to the relatively short duration of the rats to these slight temperature, humidity, and oxygen digressions, the environmental conditions within the exposure chamber were considered to

be acceptable and did not affect the validity of the study. Chamber environmental conditions are summarized in Table 4.

Table 4. Summary of Environmental Conditions in Exposure Chamber

Exposure	Temperature (°C)	Relative Humidity (%)	Airflow (L/min)	Nitrogen Flow (L/min)	Oxygen (%)
LC_{50} Exposure 1	24-25 (n=4)	28-33 (n=3)	10 (n=3)	0.6-0.8 (n=2)	19.3-19.8 (n=6)
LC_{50} Exposure 2	22-25 (n=3)	30-33 (n=3)	10 (n=3)	1.2-1.4 (n=3)	18.9-19.4 (n=4)
Multi-Time Point Blood Absorption	21 (n=3)	15-23 (n= 3)	10 (n=3)	0.6-0.9 (n=6)	19.6-20 (n=3)

Legend:
°C = degrees Centigrade
% = percent
L/min = liters per minute
n = number of samples collected

7.4 Equivalent Oral Doses

Calculated equivalent oral doses for the gavage portion of the multi-time point blood absorption study, based on body weight and average analytical concentration for the inhalation portion of the study, ranged from 112.4 – 117.8 mg/kg for the male rats and from 144.5 – 157.4 mg/kg for the female rats.

7.5 Mortality and LC_{50} Determination

All 20 animals exposed during the two acute LC_{50} exposures (five rats/sex/exposure) survived the 4-hour exposures and subsequent 14-day recovery periods. Therefore, the 4-hour inhalation LC_{50} of DNAN is greater than 2.4 mg/L. The six animals exposed as part of the inhalation portion of the multi-time point blood absorption study survived the 4-hour exposure and subsequent 1-day recovery period. Of the six animals exposed to an equivalent oral dose of DNAN for the multi-time point blood absorption study, one male and two female rats died prior to obtaining the 8-hour post-exposure blood sample and one additional female died prior to obtaining the 24-hour post-exposure blood sample.

7.6 Analysis of Blood Samples

A total of 71 blood samples were analyzed during the multi-time point blood absorption test for the presence of DNAN and DNP. Forty-two whole blood samples were analyzed for the six rats (seven time points) exposed via inhalation and 29 whole blood samples were analyzed for the six rats (six time points) exposed via oral gavage. Pre-term deaths in the group of six rats exposed orally to DNAN prevented the collection of a total of seven blood samples. Prior to study initiation, USAIPH LS personnel determined whole blood to be a sensitive and consistent matrix for determining the absorption of DNAN into the bloodstream of rats. The concentration of DNAN and its metabolite DNP in the whole blood samples is presented in Appendix F.

7.6.1 Inhalation

The mean whole blood concentration of DNAN in male rats exposed via inhalation (n=3) was 6.4 ± 1.85 micrograms per milliliter (µg/mL) following 1.1 hours of exposure, 13.5 ± 5.46 µg/mL following 2.0 to 2.1 hours of exposure, 16.7 ± 6.11 µg/mL following 4.2 to 4.3 hours of exposure, 4.3 ± 0.42 µg/mL at 4.1 hours post-exposure, 2.4 ± 2.44 µg/mL at 8.0 to 8.1 hours post-exposure, and 0.1 ± 0.19 µg/mL at 23.3 to 23.5 hours post-exposure. The mean whole blood concentration of DNAN in female rats exposed via inhalation (n=3) was 8.2 ± 1.65 µg/mL following 1.0 to 1.1 hours of exposure, 13.0 ± 2.65 µg/mL following 2.0 to 2.1 hours of exposure, 12.5 ± 4.80 µg/mL following 4.2 hours of exposure, 4.3 ± 3.01 µg/mL at 4.0 to 4.2 hours post-exposure, 1.6 ± 1.17 µg/mL at 8.0 to 8.1 hours post-exposure, and 0.9 ± 1.01 µg/mL at 23.3 to 23.4 hours post-exposure. DNAN was not detected in the pre-exposure (baseline) male and female blood samples taken approximately 2 hours prior to exposure. The mean whole blood concentration of DNP in male rats exposed via inhalation (n=2) was 0.0 ± 0.00 µg/mL following 1.1 hours of exposure, 26.0 ± 4.24 µg/mL following 2.0 to 2.1 hours of exposure, 44.0 ± 5.66 µg/mL following 4.2 to 4.3 hours of exposure, 51.0 ± 7.07 µg/mL at 4.1 hours post-exposure, 43.5 ± 10.61 µg/mL at 8.0 to 8.1 hours post-exposure, and 14.5 ± 2.12 µg/mL at 23.3 to 23.5 hours post-exposure. The mean whole blood concentration of DNP in female rats exposed via inhalation (n=3) was 29.0 ± 8.54 µg/mL following 1.0 to 1.1 hours of exposure, 59.0 ± 11.53 µg/mL following 2.0 to 2.1 hours of exposure, 71.3 ± 9.50 µg/mL following 4.2 hours of exposure, 70.7 ± 17.01 µg/mL at 4.0 to 4.2 hours post-exposure, 56.3 ± 13.58 µg/mL at 8.0 to 8.1 hours post-exposure, and 29.3 ± 4.62 µg/mL at 23.3 to 23.4 hours post-exposure. With the exception of one male rat excluded from analysis, DNP was not detected in the pre-exposure (baseline) male and female blood samples taken approximately 2 hours prior to exposure.

7.6.2 Oral

The mean whole blood concentration of DNAN in orally-gavaged male rats was 25.3 ± 19.66 µg/mL (n=3) at 1.0 to 1.1 hours post-exposure, 17.0 ± 14.82 µg/mL (n=3) at 2.0 to 2.1 hours post-exposure, 16.3 ± 15.44 µg/mL (n=3) at 4.1 to 4.2 hours post-exposure, 9.9 ± 8.63 µg/mL (n=2) at 8.1 hours post-exposure, and 0.3 ± 0.06 µg/mL (n=2) at 24.6 hours post-exposure. The mean whole blood concentration of DNAN in orally-gavaged female rats was 36.0 ± 3.00 µg/mL (n=3) at 1.0 to 1.1 hours post-exposure, 32.3 ± 5.69 µg/mL (n=3) at 2.0 to 2.1 hours post-exposure, 48.0 ± 9.54 µg/mL (n=3) at 4.0 to 4.1 hours post-exposure, and 45.0 ± 0.00 µg/mL (n=1) at 7.4 hours post-exposure. All orally-gavaged female rats died prior to the 24-hour post-exposure blood sample. DNAN was not detected in the pre-exposure (baseline) male and female blood samples taken several minutes prior to dosing.

The mean whole blood concentration of DNP in orally-gavaged male rats was 31.0 ± 2.65 µg/mL (n=3) at 1.0 to 1.1 hours post-exposure, 47.3 ± 13.58 µg/mL (n=3) at 2.0 to 2.1 hours post-exposure, 51.3 ± 13.80 µg/mL (n=3) at 4.1 to 4.2 hours post-exposure, 50.0 ± 4.24 µg/mL (n=2) at 8.1 hours post-exposure, and 19.5 ± 3.54 µg/mL (n=2) at 24.6 hours post-exposure. The mean whole blood concentration of DNP in orally-gavaged female rats was 33.7 ± 5.69 µg/mL (n=3) at 1.0 to 1.1 hours post-exposure, 50.0 ± 8.72 µg/mL (n=3) at 2.0 to 2.1 hours post-exposure, 58.0 ± 6.00 µg/mL (n=3) at 4 to 4.1 hours post-exposure, and 54.0 ± 0.00 µg/mL (n=1) at 7.4 hours post-exposure. All orally-gavaged female rats died prior to the 24-hour post-exposure blood sample. DNP was not detected in the pre-exposure (baseline) male and female blood samples taken several minutes prior to dosing.

7.7 Analysis of Urine Samples

The mean urine concentration of DNAN in rats exposed via inhalation was 8.0 ± 6.86 µg/mL for male rats (n=3) and 11.1 ± 10.36 µg/mL for female rats (n=3). The mean urine concentration of DNP in rats exposed via inhalation was 45.1 ± 73.60 µg/mL for male rats (n=3) and 3.0 ± 5.20 µg/mL for female rats (n=3). The mean urine concentration of DNAN in orally-gavaged rats was 1.1 ± 1.09 µg/mL for male rats (n=2) while the DNP concentration was 37.6 ± 50.13 µg/mL for male rats (n=2). DNAN and DNP urine concentrations in orally-gavaged female rats could not be determined due to pre-term deaths. The concentration of DNAN and DNP in the urine samples is presented in Appendix G.

7.8 Body Weights of Rats

Rats exposed as part of the first LC_{50} inhalation exposure were weighed on test days 1, 2, 3, 6, 8, 13, and 15. With the exception of one male and two female rats, all other rats either maintained their pre-exposure body weight or gained weight by post-exposure day 1. The weight loss of these three rats averaged 1-2 percent, however, all animals experienced normal weight gain by post-exposure day 2.

Rats exposed as part of the second LC_{50} inhalation exposure were weighed on test days 1, 2, 3, 7, 10, and 15. Two out of five male rats exhibited a 1-2 percent weight loss and four out of five female rats exhibited a 1-5 percent weight loss on post-exposure day 1 but all animals began to gain weight at a normal rate by post-exposure day 2.
All rats exposed as part of the multi-time point blood absorption test, both inhalation and gavage, were weighed prior to exposure and on the day following exposure. All rats exposed via inhalation except one female exhibited a 1-9 percent body weight loss on post-exposure day 1. The two surviving male rats exposed via oral gavage exhibited a 6-9 percent weight loss on post-exposure day 1. The individual body weights for all rats are reported in Appendix H.

7.9 Clinical Observations of Rats

Rats were generally observed approximately every hour during each of the inhalation exposures. During the exposures, most rats generally began to form a yellow crust around their noses and on their whiskers within the first hour of the exposure. The yellow crust remained throughout the exposure but did not appear to effect respiration. Additional signs observed upon removal from the exposure chamber included yellow-stained fur on the face and head, salivation, and red-colored discharge around the nose and/or eyes. With the exception of yellow facial staining, most of the post-exposure observations disappeared by first day of the post-exposure observation period. The yellow facial staining from the test compound typically persisted throughout the post-exposure observation period. In addition to the signs described above, one male and one female rat exposed as part of the inhalation portion of the multi-time point blood absorption test were noted to be slightly dragging their left rear leg during the 24-hour post-exposure period. Observations noted for the rats dosed via oral gavage for the multi-time point blood absorption study were more severe and included the pre-term deaths of one out of three males and three out of three females. Periods of prostrate posture, lethargy, and increased/elevated respiration typically preceded death. All three female rats also had their back limbs splayed behind them. Other signs noted in animals gavaged with DNAN included tearing of the eyes, yellow-stained bedding, and red-colored discharge around nose/eyes. The individual clinical signs for all rats are reported in Appendix I.

7.10 Pathology

All animals exposed during the two LC_{50} exposures had minimal to mild yellow staining on various parts of the head, chest, and/or forearms at the time of gross necropsy. One male rat exposed during the first LC_{50} exposure also had a mildly enlarged spleen. One male rat exposed during the second LC_{50} exposure had mottled lungs with froth in the lower trachea and a second male rat had a slightly enlarged spleen. Rats exposed for the multi-time point blood absorption test did not have a gross necropsy performed following the final blood sample. The individual gross necropsy findings for LC_{50} rats are reported in Appendix J.

7.11 Statistics

All statistics were performed on the 4-hour cumulative AUC values due to pre-term deaths experienced after the 4 hour bleed time. In addition, DNP blood concentrations from rat number 12-0071 were excluded from analysis due to the apparent presence of DNP in the baseline blood sample. The ANOVA performed on the DNAN 4-hour cumulative AUC's indicated that the oral route of administration resulted in higher DNAN blood concentrations than the inhalation route of administration ($p=0.019$). Male and female DNAN blood concentrations were not different, regardless of route of administration ($p=0.161$). Analysis of the DNP 4-hour cumulative AUC's showed that female rats had higher blood concentrations compared to male rats ($p=0.041$). DNP blood concentrations from rats administered DNAN orally did not differ from those exposed to DNAN via inhalation ($p=0.423$). For the oral route of administration, the cumulative 4-hour DNP blood concentrations were higher than the 4-hour DNAN blood concentrations ($p=0.015$). DNAN and DNP blood concentrations of orally-exposed female rats were not different from male rats ($p=0.189$). For those animals exposed via inhalation, female rats exhibited higher DNAN and DNP blood concentrations compared to male rats ($p=0.040$) and DNP blood concentrations were elevated compared to DNAN blood concentrations ($p=0.003$).

8 Discussion

This study was conducted to determine the 4-hour, inhalation LC_{50} of DNAN in male and female rats. Previous attempts at other toxicology laboratories to generate DNAN as a vapor/aerosol resulted in extremely low chamber concentrations so the DNAN was dissolved and generated in acetone to obtain acute inhalation toxicity data. This testing of DNAN aerosolized in acetone resulted in a 4-hour LC_{50} in rats of > 3 mg/L. Since the use of acetone introduced some uncertainty into the previously reported LC_{50} value, all method development work for this current study was conducted using neat DNAN with the intention of generating 2 mg/L for an acute inhalation limit test. Under the limit test provision of the EPA test guidelines for materials that are not expected to be acutely toxic via inhalation, a single group of five male and five female rats are exposed to a 2 mg/L test atmosphere for 4 hours. If no lethality is demonstrated at 2 mg/L, no further testing for acute inhalation toxicity is needed (EPA, 1998). Repeated attempts during method development work to generate a DNAN aerosol/vapor atmosphere of 2 mg/L at the appropriate particle size were unsuccessful, so the design concentration of the first LC_{50} exposure was approximately 1 mg/L. Changes in the generation system at the conclusion of the first LC_{50} exposure revealed that higher chamber concentrations could be achieved so the LC_{50} exposure was repeated at a design concentration of approximately 2 mg/L. Since no mortalities resulted from the 4-hour exposure to 2.4 mg/L DNAN, the LC_{50} will be reported as >2.4 mg/L.

A secondary objective of this study was to determine the effect that two different routes of administration (inhalation and oral) had on the absorption of DNAN into the bloodstream as well as

its conversion to DNP. DNAN was reported to be moderately toxic with an acute oral LD_{50} value of 199 mg/kg (Dodd and McDougal, 2002). A series of oral toxicity studies performed by this Institute (acute, subacute, and subchronic) confirmed that DNAN was moderately acutely toxic via oral gavage with lethality reported at doses of 300 mg/kg and above (USAPHC, 2012). The intent of the oral toxicity work was to derive an inhalation OEL from the data, however it was previously unknown if differences existed between the absorption of DNAN into the blood through the respiratory and gastrointestinal tracts. The design concentration of the inhalation exposure for the multi-time point blood absorption test was determined so that the equivalent oral dose would be approximately half of the dose that previously induced lethality (150 mg/kg). After calculating the equivalent oral doses taking into account the actual concentration of the inhalation exposure and the body weight of the orally-exposed animals, doses for the male rats ranged from 112 to 118 mg/kg and doses for female rats ranged from 145 to 157 mg/kg. It is believed that the pre-term deaths experienced in the rats dosed by oral gavage (one male, three female) may have been experienced at lower doses than anticipated due to the repeated blood samples taken following exposure. Although only 200 microliters of blood was taken at each time point, the number of time points combined with the relatively low circulating blood volume of laboratory rats (approximately 54 mL/kg) may have induced DNAN toxicity at lower doses than previously experienced. In addition, certain clinical signs of toxicity (dragging rear limbs) were observed in several rats following the inhalation portion of the multi-time point test (0.86 mg/L) that were not observed following the second LC_{50} exposure (2.39 mg/L) and this was also attributed to the repeated blood samples collected from these rats.

It is understood that the previously reported calculation to derive an equivalent oral dose from an inhalation exposure has a number of uncertainties associated with it. First, the amount of DNAN retained in the entire respiratory system (α), was assumed to be approximately 90 percent of the analytical chamber concentration. A number of factors, including the chemical properties of the test substance, uptake from the upper respiratory pathways versus the lungs, deposition in the lungs, and the particle size of the generated test material, will greatly influence the amount of test material retained in the respiratory system. Every attempt was made to generate the DNAN with an MMAD in the range considered respirable for rats (1-4 µm), however the MMAD for the absorption study ranged from 4.6 to 4.9 µm. The larger particles contained in a test atmosphere are typically retained in the mucous-lined head region and tracheobronchial airways where they may be absorbed or transported out of the respiratory airways via ciliary action. The particles transported out of the airways may then either be expelled or swallowed to the gastrointestinal tract, essentially leading to an additional oral dose. The smaller, soluble particles reaching the alveolar (deep lung) regions may then be absorbed into the blood. Based on the particle size alone, the assumption that 90 percent of the DNAN aerosol was retained in the respiratory system may have been a high estimate. However, a certain percentage of larger particles could have been absorbed in the upper respiratory pathways or transported out of the respiratory pathways and swallowed, leading to an increased amount of absorption. Second, an average minute volume of 0.00016 m^3 for rats was used for all animals. Although the resulting variations in the calculated oral dose would be miniscule, the changes in minute volume based on animal size, sex, and respiratory rate were not accounted for in the calculations. Third, the calculation used to determine the equivalent oral dose was not designed to be used with the gavage method of oral administration due to the bolus effect. The rats exposed via inhalation for the multi-time point blood absorption test were exposed to a consistent concentration for a period of 4 hours while rats exposed via oral gavage received the entire dose at one time. Obviously differences in the method of dose administration will lead to differences in the blood concentration/time curves; however, gavage is still one of the most accurate methods to administer an oral dose. Alternative methods to slowly introduce the test substance into the rats (i.e., timed injection) would have involved the introduction of a different route of exposure and the blood concentrations resulting from an oral dose were most desirable

since the current proposed OEL was extrapolated from the results of an oral subchronic study. Mixing the test substance in animal feed was not considered an option for this study due to the inaccuracy of the dose (i.e., food spillage) and difficulties ensuring that the animals consumed all of the food within a specified period of time. In addition, due to the pre-term deaths in rats exposed to DNAN via oral gavage, statistical analysis of the cumulative AUC's was performed on the 4-hour blood samples. For those animals exposed via inhalation, this blood sample would have been taken immediately upon removal from the exposure chamber while the blood sample would have been taken 4-hours following dose administration for those animals orally-gavaged.

Given the inherent limitations of comparing the whole blood concentrations of a test material introduced by two different exposure routes, acute exposure to DNAN via oral gavage does appear to induce higher DNAN blood concentrations in laboratory rats compared to those exposed via inhalation. Four-hour cumulative AUC blood values for rats exposed via inhalation exhibited a lower average of 41.73 ± 12.01 µg/mL compared to the average of 99.81 ± 51.75 µg/mL for orally-gavaged rats. Average DNAN blood concentrations peaked at the 1-hour sampling time for male rats orally dosed. The average DNAN blood concentrations peaked at the 4-hour sampling time for those animals exposed via inhalation and for female rats dosed orally, which was somewhat unexpected given that this time point represented 4 hours following dosing for orally gavaged animals and was immediately upon removal from the exposure chamber for those animals exposed via inhalation. Average DNAN blood concentrations for female animals exposed via gavage initially appeared to peak at 1-hour post-exposure sample since the concentrations dropped at the 2-hour post-exposure sample but then increased and peaked at the 4-hour sample. These average blood concentrations generally began to decrease between 4 and 8 hours post-exposure and were nearly undetectable at 24 hours. Cumulative average 4-hour AUC values for male and female rats were similar for rats exposed via inhalation (43.30 and 40.17 µg/mL, respectively) while these 4-hour averages for female rats exposed orally were nearly double those of male rats (132.50 and 67.12 µg/mL, respectively).

DNP blood concentrations followed the same general trends as the DNAN blood concentrations. Four-hour cumulative area-under-curve blood values averaged 150.83 ± 56.80 µg/mL for rats exposed by inhalation and 160.00 ± 25.70 µg/mL for rats exposed by oral gavage. Average DNP blood concentrations peaked at the 4-hour sampling time for both methods of exposure but did not decrease during the post-exposure period as rapidly as DNAN. Average DNP blood concentrations for animals exposed via gavage decreased approximately 6 percent between 4 and 8 hours post-exposure while these average blood concentrations decreased approximately 18 percent for those animals exposed via inhalation. At 24-hours post-exposure, average DNP blood concentrations were 23.00 and 19.50 µg/mL for animals exposed via inhalation compared to oral gavage. In contrast to DNAN, cumulative average DNP 4-hour AUC values for male and female rats were similar for rats exposed via oral gavage (153.33 and 166.67 µg/mL, respectively) while these 4-hour averages for female rats exposed via inhalation were higher than those of male rats (188.83 and 112.83 µg/mL, respectively).

Although the DNAN and DNP whole blood concentrations observed between male and female rats exposed via inhalation and oral gavage were statistically compared, the fact that male and female rats are typically exposed simultaneously to the same test concentration during acute inhalation studies likely led to the elevated female blood concentrations compared to the males. By study design, the female rats essentially receive a higher dose per body weight since they are exposed to the same test atmosphere concentration. The calculation used for converting an inhalation dose to an oral dose does consider body weight but with the males and females exposed to identical inhalation atmospheres, the conversion calculation also yields a higher oral mg/kg dose. The results of this study do suggest that female rats convert a greater proportion of DNAN to DNP than

male rats when exposed via inhalation while male rats convert a greater proportion of DNAN to DNP than female rats when exposed orally.

9 Conclusions

Rats were exposed nose-only to a 2.4 mg/L aerosol atmosphere of DNAN for a single 4-hour exposure. No test compound-related mortalities occurred in rats exposed during the inhalation phase of the study and no adverse toxic signs, body weight changes, or gross necropsy findings were observed in exposed rats. The LC_{50} portion of this study indicates that acute inhalation exposure to the highest-achievable concentration of DNAN aerosol (2.4 mg/L) is relatively nontoxic to rats.

The results of the multi-time point blood absorption portion indicated that, under the stated study conditions and limitations, acute exposure to DNAN via oral gavage appears to induce higher DNAN whole blood concentrations in laboratory rats compared to those exposed via inhalation. Blood concentrations of the metabolite DNP were not different between the oral and inhalation routes of exposure. Female rats appear to convert a greater proportion of DNAN to DNP than male rats when exposed via inhalation while male rats convert a greater proportion of DNAN to DNP than female rats when exposed orally.

10 Point of Contact

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Portfolio Director, Toxicology

17-MAR-2015
Date

Appendix A

References

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Appendix B

Quality Assurance Statement

QUALITY ASSURANCE STATEMENT

For: Toxicology Study No. 85-XC-0ENTa-11, Protocol No. 0ENT-24-11-07-03, Acute Inhalation Toxicity and Blood Absorption of 2,4-Dinitroanisole (DNAN) in Rats, the following critical phases were inspected/audited by the Quality Systems Office (QSO):

Critical Phase Inspected/Audited	Date Inspected /Audited	Date Reported to Management/SD
Study Protocol Good Laboratory Practice Standards and Animal Care Review	05/26/2011	05/26/2011

Critical Phase Inspected/Audited	Date Inspected /Audited	Date Reported to Management/SD
LC50/Rangefinding-Inhalation Exposure Duration	11/16/2011	11/28/2011
LC50/Rangefinding-Test Article Control & Administration	11/16/2011	11/28/2011
LC50/Rangefinding-Test System ID & Restrainer Procedure	11/16/2011	11/28/2011
LC50/Rangefinding-Analysis of the Test Atmosphere	11/29/2011	12/07/2011
Rangefinding Blood Absorption - Euthanasia procedure	11/29/2011	12/07/2011
Rangefinding Blood Absorption - Blood Collection	11/29/2011	12/07/2011
Inhalation Exp # 3 Recovery Period Duration	12/13/2011	12/22/2011
Inhalation Exp # 3 Gross Necropsy Procedures	12/13/2011	12/22/2011
Experiment 3 - Oral Gavage Test Substance Administration	12/16/2011	12/22/2011
Experiment 3 - Gavage Pre-Bleed Catheter Collection	12/16/2011	12/22/2011
Experiment 3 - Urine Collection/Compliance w/ Study Mod # 2	12/16/2011	12/22/2011
In-Life Study Endpoint Criteria	12/16/2011	12/22/2011
Re-inspection of corrective action for CPI report # 111222-03	02/15/2012	03/02/2012
Final Study Report Review	05/17/2013	05/21/2013
Study Raw Data Review	05/17/2013	05/21/2013

Note: All findings were made known to the Study Director and the Program Manager at the time of the audit/inspection. If there were no findings during the inspection, the inspection was reported to Management and the Study Director on the date shown in the table. A review of the raw data and records indicates the above mentioned report accurately reflects the raw data and study as it was conducted.

Michael P. Kefauver
GLP Quality Assurance Specialist, QSO

03/06/2015
Date

Appendix C

Archives and Study Personnel

1. ARCHIVES.

a. All raw data, documentation, records, protocol, and a copy of the final report generated as a result of this study will be archived in room 1026, Building E-2100, USAPHC, for a minimum of five (5) years following submission of the final report to the Sponsor.

b. Records on animal receipt, diet, and facility environmental parameters will be archived by the Veterinary Medical Division, Toxicology Portfolio, for a minimum of five (5) years following submission of the final report to the Sponsor.

c. Some ancillary records pertaining to this study, such as instrument maintenance logs, animal room observation logs, etc., will not be archived until those logbooks have been completed. Once complete they will be archived in room 1026, Building E-2100, USAPHC.

d. Wet tissues, histology slides, and paraffin blocks are stored in building E-5158, if applicable.

2. PERSONNEL.

a. Management.

(1) Management (In-Life): COL Chris E. Hanson, Portfolio Director, Toxicology; Glenn J. Leach, Ph.D., Program Manager, Toxicity Evaluation Program (TEP); Dr. Mark S. Johnson, Ph.D., Program Manager, Health Effects Research Program (HERP).

(2) Management (Report): Mark S. Johnson, Portfolio Director, Toxicology; Arthur J. O'Neill, Program Manager, Toxicity Evaluation Program (TEP); Dr. Michael J. Quinn, Ph.D., Program Manager, Health Effects Research Program (HERP).

b. Study Director: Arthur J. O'Neill, Biologist, Toxicity Evaluation Program (TEP).

c. Quality Assurance: Michael P. Kefauver, Quality Assurance Specialist, Quality Systems Office.

d. Veterinary Support and Animal Care: Dawn C. Fitzhugh, DVM, LTC, VC; Robert Sunderland, Animal Health Technician; Rebecca Kilby, Animal Health Technician; Jason Williams, Animal Health Technician.

e. Pathology Lab Coordinator: Patricia A. Beall, Biologist, TEP.

f. Histopathology: Shannon M. Wallace, DVM, DACVP, LTC, VC, Pathologist, VMD.

g. In-Life Support: Lee C.B. Crouse, Biologist, TEP.

h. Hematology, Clinical Chemistry, Urinalysis: Matthew A. Bazar, Biologist, TEP; Mark R. Way, Biologist, TEP.

i. Archivist: Martha L. Thompson, Data Acquisition Specialist, TEP.

Appendix D

Chamber Distribution

APPENDIX D
Protocol No. 0ENT-24-11-07-03
Acute Inhalation Toxicity and Blood Absorption of DNAN in Rats

Chamber Distribution of DNAN Aerosol

Location of Sample Port	Sample #	Concentration of DNAN (mg/L)
Reference Port	1	0.940
Reference Port	3	0.981
Reference Port	5	0.982
Reference Port	7	0.932
	Mean	**0.959**
Top Right of Faceplate	2	0.972
Middle Center of Faceplate	4	0.990
Bottom Left of Faceplate	6	0.996
	Mean	**0.986**
Total of 7 Samples	**Mean**	**0.970**

The reference port was designated side port of the exposure chamber.

Confirmation of Uniform Distribution of DNAN Aerosol Within Exposure Chamber

Top Right of Faceplate	0.972 mg/L /0.986 mg/L = 99%
Middle Center of Faceplate	0.990 mg/L / 0.986 mg/L = 100%
Bottom Left of Faceplate	0.996 mg/L / 0.986 mg/L = 101%

The first and last rows of faceplate ports were not used for the animal exposures due to the lack of aerosol uniformity.

Confirmation of Correlation Between Reference Sample Port and Animal Exposure Ports

Reference Faceplate Port Sample #1	0.940 mg/L / 0.986 mg/L = 95%
Reference Faceplate Port Sample #2	0.981 mg/L / 0.986 mg/L = 99%
Reference Faceplate Port Sample #3	0.982 mg/L / 0.986 mg/L = 100%
Reference Faceplate Port Sample #4	0.932 mg/L / 0.986 mg/L = 95%

Appendix E

Exposure Chamber Atmospheric Concentration

APPENDIX E
Protocol No. 0ENT-24-11-07-03
Acute Inhalation Toxicity and Blood
Absorption of DNAN in Rats

Exposure Chamber Atmospheric Concentration

Acute LC_{50} Test (0.7 mg/L)

	DNAN Aerosol Concentrations (mg/L)		DNAN Vapor Concentrations (mg/L)
Sample #	Gravimetric (Filter)	Analytical (Filter)*	Analytical (Xad-2 Tube)*
1	0.702	0.6	0.00018
2	0.684		
3	0.695		
4	0.625		
5	0.591		
6	0.562	0.5	0.00004
7	0.844		
8	0.839		
9	0.814		
10	0.838		
11	0.793	0.8	0.00004
Mean ± S.D.	**0.7261 ± 0.10493**	**0.63 ± 0.153**	**0.000087 ± 0.0000808**

Acute LC_{50} Test (2.4 mg/L)

	DNAN Aerosol Concentrations (mg/L)		DNAN Vapor Concentrations (mg/L)
Sample #	Gravimetric (Filter)	Analytical (Filter)*	Analytical (Xad-2 Tube)*
1	2.690	2.3	0.00065
2	1.843		
3	2.610		
4	2.639		
5	2.390	1.9	0.00004
6	2.400		
7	2.320		
8	2.199		
9	2.400		
10	2.390	2.1000	0.00007
Mean ± S.D.	**2.3881 ± 0.24521**	**2.10 ± 0.200**	**0.000253 ± 0.0003439**

Multi-Time Point Blood Absorption Test

	DNAN Aerosol Concentration (mg/L)
Sample #	Gravimetric (Filter)
1	0.622
2	0.646
3	0.796
4	0.725
5	0.832
6	0.845
7	0.757
8	1.086
9	1.02
10	0.902
11	0.838
12	0.797
13	0.795
14	1.11
15	1.07
Mean ± S.D.	**0.8561 ± 0.15350**

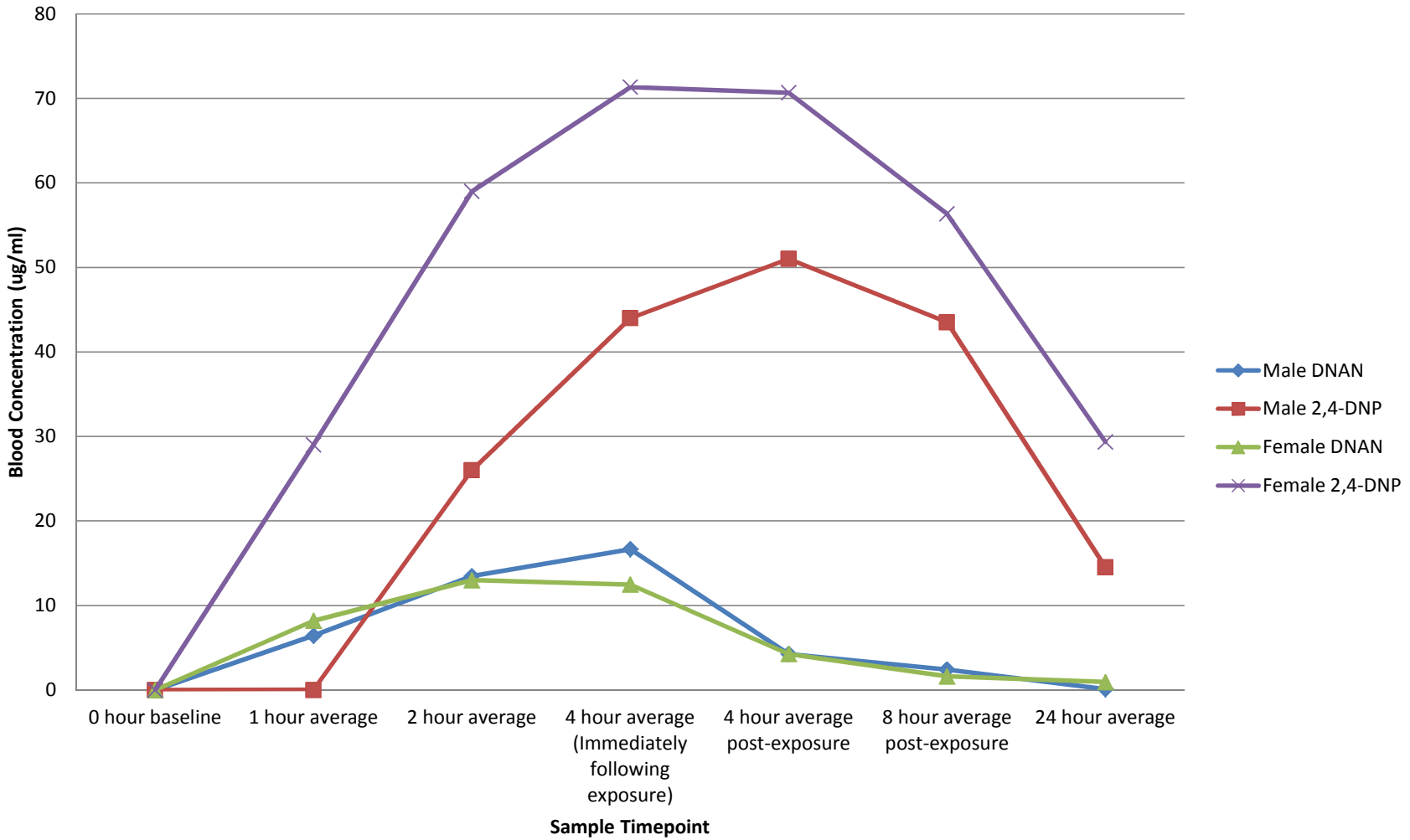
* = selected time-points for LC_{50} tests only

Appendix F

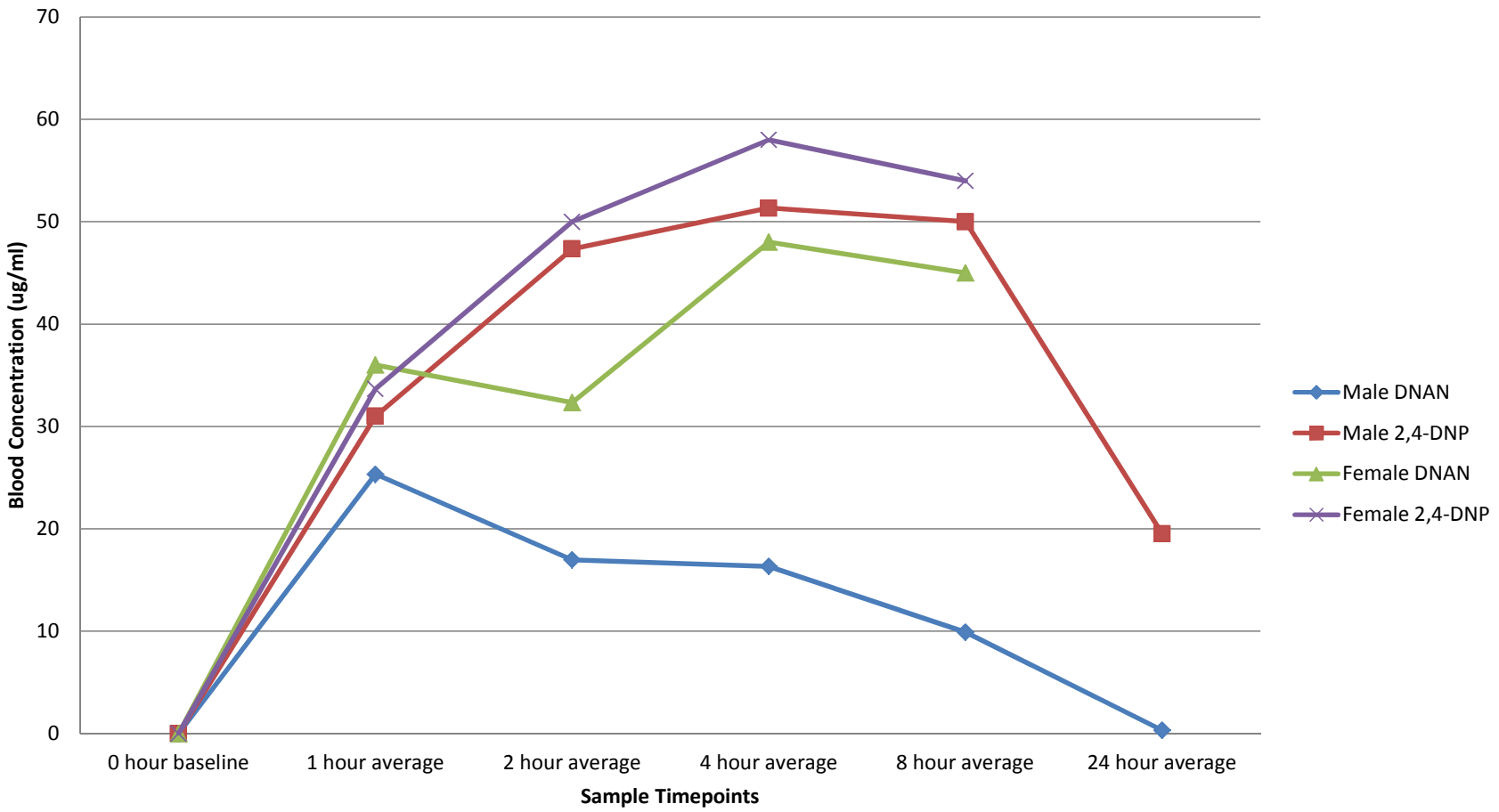
Blood Concentrations

Appendix F
Protocol No. 0ENT-24-11-07-03
Acute Inhalation Toxicity and Blood Absorption of DNAN in Rats

Average DNAN and 2,4-DNP Inhalation Blood Concentrations



Appendix F
Protocol No. 0ENT-24-11-07-04
Acute Inhalation Toxicity and Blood Absorption of DNAN in Rats



Note: Due to pre-term mortality, the 8 hour average included 2/3 male rats and 1/3 female rats while the 24 hour average included 2/3 male rats and 0/3 female rats.

APPENDIX F
Protocol No. 0ENT-24-11-07-03
Acute Inhalation Toxicity and Blood
Absorption of DNAN in Rats

Individual Blood Concentrations For
Multi-Time Point Blood Absorption Test

Inhalation

			DNAN Blood Concentrations (ug/ml)						
Animal #	Sex	Exposure Level	0 Hour (Baseline)	1 Hour	2 Hour	4 Hour[a]	8 Hour[b]	12 Hour[c]	24 Hour[d]
12-0069	Male	.856 mg/L	0	7.5	15	18	3.8	0.66	0
12-0070	Male	.883 mg/L	0	4.3	7.4	10	4.4	5.2	0
12-0071	Male	.904 mg/L	0	7.5	18	22	4.6	1.4	0.33
12-0076	Female	.873 mg/L	0	6.3	11	10	2.1	0.62	0.27
12-0077	Female	.890 mg/L	0	9.2	16	18	7.7	2.9	2.1
12-0078	Female	.901 mg/L	0	9.1	12	9.4	3	1.3	0.46
		Mean	**0**	**7.32**	**13.23**	**14.57**	**4.27**	**2.01**	**0.53**
		S.D.	**0**	**1.840**	**3.848**	**5.426**	**1.920**	**1.766**	**0.792**

			2,4-DNP Blood Concentrations (ug/ml)						
Animal #	Sex	Exposure Level	0 Hour (Baseline)	1 Hour	2 Hour	4 Hour[a]	8 Hour[b]	12 Hour[c]	24 Hour[d]
12-0069	Male	.856 mg/L	0	0	29	48	56	36	16
12-0070	Male	.883 mg/L	0	0	23	40	46	51	13
12-0071	Male	.904 mg/L	15*	24*	47*	63*	45*	39*	21*
12-0076	Female	.873 mg/L	0	21	47	62	54	42	24
12-0077	Female	.890 mg/L	0	28	60	81	88	69	32
12-0078	Female	.901 mg/L	0	38	70	71	70	58	32
		Mean	**0.00**	**17.40**	**45.80**	**60.40**	**62.80**	**51.20**	**23.40**
		S.D.	**0.000**	**16.994**	**19.942**	**16.652**	**16.529**	**13.027**	**8.820**

a. Represents blood samples taken immediately following removal from exposure chamber.
b. Represents blood samples taken 4 hours following inhalation exposure.
c. Represents blood samples taken 8 hours following inhalation exposure.
d. Represents blood samples taken the following morning after inhalation exposure.
* = Samples were flagged and excluded due to possible cross-contamination.

Oral Gavage

			DNAN Blood Concentrations (ug/ml)					
Animal #	Sex	Equivalent Oral Dose	0 Hour (Baseline)	1 Hour	2 Hour	4 Hour	8 Hour	24 Hour
12-0072	Male	112.4 mg/kg	0	48	34	34	ND	ND
12-0073	Male	113.2 mg/kg	0	15	9.9	5.4	3.8	0.27
12-0074	Male	117.8 mg/kg	0	13	7	9.6	16	0.36
12-0079	Female	157.4 mg/kg	0	39	26	37	ND	ND
12-0080	Female	144.5 mg/kg	0	33	37	54	45	ND
12-0081	Female	156.6 mg/kg	0	36	34	53	ND	ND
		Mean	**0**	**30.67**	**24.65**	**32.17**	**21.60**	**0.32**
		S.D.	**0**	**13.866**	**13.102**	**20.800**	**21.163**	**0.064**

			2,4-DNP Blood Concentrations (ug/ml)					
Animal #	Sex	Equivalent Oral Dose	0 Hour (Baseline)	1 Hour	2 Hour	4 Hour	8 Hour	24 Hour
12-0072	Male	24.2 mg/kg	0	28	60	67	ND	ND
12-0073	Male	24.6 mg/kg	0	32	49	46	53	22
12-0074	Male	24.5 mg/kg	0	33	33	41	47	17
12-0079	Female	34.7 mg/kg	0	40	60	64	ND	ND
12-0080	Female	38.2 mg/kg	0	29	46	58	54	ND
12-0081	Female	35.6 mg/kg	0	32	44	52	ND	ND
		Mean	**0**	**32.33**	**48.67**	**54.67**	**51.33**	**19.50**
		S.D.	**0**	**4.227**	**10.309**	**10.191**	**3.786**	**3.536**

ND = No data. Animal died while on study.

APPENDIX F (cont)
Protocol No. 0ENT-24-11-07-03
Acute Inhalation Toxicity and Blood Absorption of DNAN in Rats

Summary of Blood Concentrations For Multi-Time Point Blood Absorption Test

Inhalation

			DNAN Blood Concentrations (ug/ml)						
Sex	Exposure Level		0 Hour (Baseline)	1 Hour	2 Hour	4 Hour[a]	8 Hour[b]	12 Hour[c]	24 Hour[d]
Male	.922 mg/L	Mean	0	6.43	13.47	16.67	4.27	2.42	0.11
		S.D.	0	1.848	5.464	6.110	0.416	2.436	0.191
Female	.922 mg/L	Mean	0	8.20	13.00	12.47	4.27	1.61	0.94
		S.D.	0	1.646	2.646	4.801	3.007	1.171	1.006

			2,4-DNP Blood Concentrations (ug/ml)						
Sex	Exposure Level		0 Hour (Baseline)	1 Hour	2 Hour	4 Hour[a]	8 Hour[b]	12 Hour[c]	24 Hour[d]
Male	.922 mg/L	Mean	0	0	26.00	44.00	51.00	43.50	14.50
		S.D.	0	0	4.243	5.657	7.071	10.607	2.121
Female	.922 mg/L	Mean	0	29.00	59.00	71.33	70.67	56.33	29.33
		S.D.	0	8.544	11.533	9.504	17.010	13.577	4.619

a. Represents blood samples taken immediately following removal from exposure chamber.
b. Represents blood samples taken 4 hours following inhalation exposure.
c. Represents blood samples taken 8 hours following inhalation exposure.
d. Represents blood samples taken the following morning after inhalation exposure.

Oral Gavage

	Average		DNAN Blood Concentrations (ug/ml)					
Sex	Equivalent Oral Dose		0 Hour (Baseline)	1 Hour	2 Hour	4 Hour	8 Hour	24 Hour
Male	114.5 mg/kg	Mean	0	25.33	16.97	16.33	9.90	0.32
		S.D.	0	19.655	14.822	15.443	8.627	0.064
Female	152.8 mg/kg	Mean	0	36.00	32.33	48.00	45.00	ND
		S.D.	0	3.000	5.686	9.539	ND	ND

	Average		2,4-DNP Blood Concentrations (ug/ml)					
Sex	Equivalent Oral Dose		0 Hour (Baseline)	1 Hour	2 Hour	4 Hour	8 Hour	24 Hour
Male	114.5 mg/kg	Mean	0	31.00	47.33	51.33	50.00	19.50
		S.D.	0	2.646	13.577	13.796	4.243	3.536
Female	152.8 mg/kg	Mean	0	33.67	50.00	58.00	54.00	ND
		S.D.	0	5.686	8.718	6.000	ND	ND

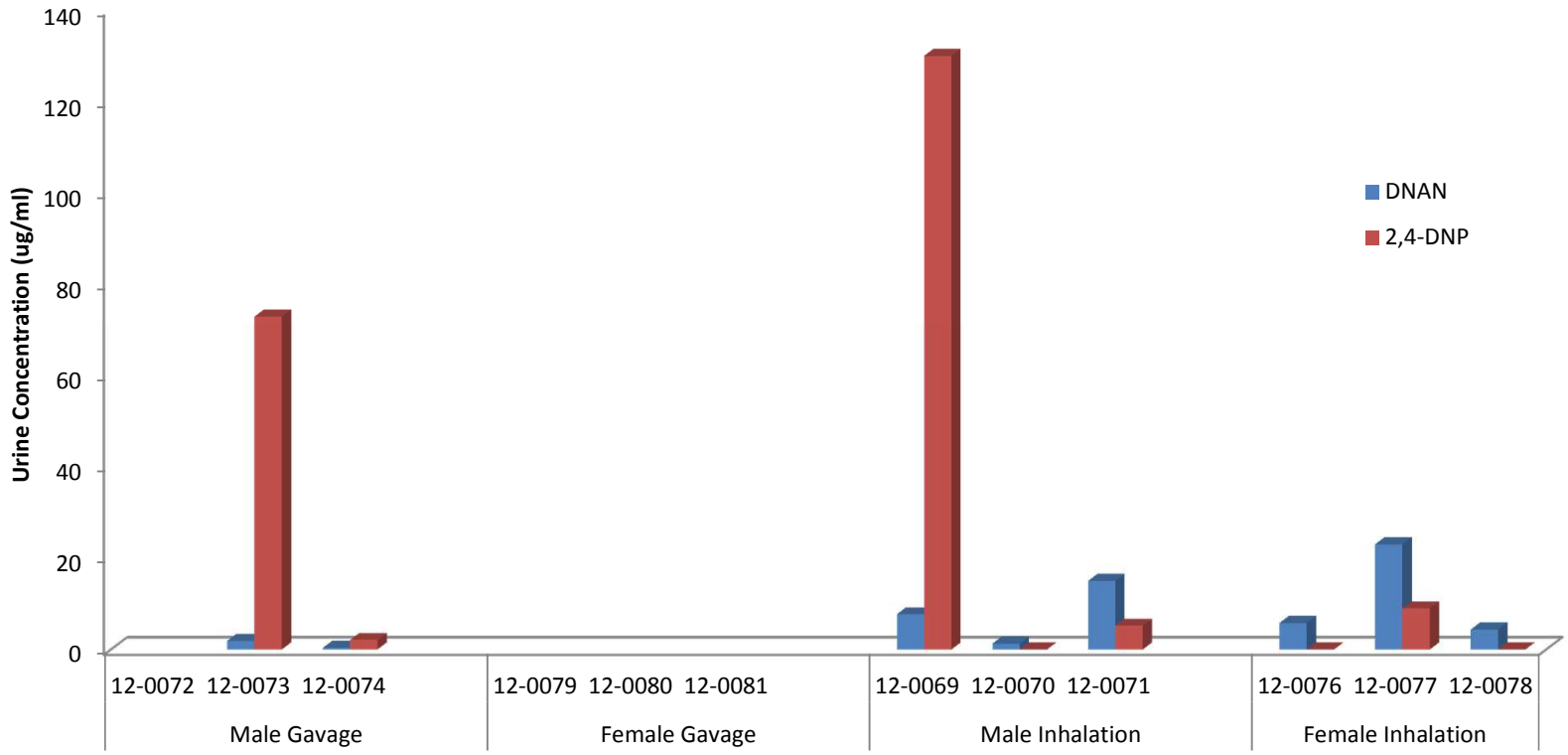
ND = No data. Animal died on study.

Appendix G

Urine Concentrations

Appendix G
Protocol No. 0ENT-24-11-07-03
Acute Inhalation Toxicity and Blood Absorption of DNAN in Rats

DNAN Urine Concentrations Gavage vs. Inhalation



Note: No samples were collected for animal number 12-0072, 12-0079, 12-0080, and 12-0081. These animals died prior to being placed in the metabolism cages. All remaining animals were placed in the metabolism cages overnight on the day of exposure and collected the following morning for analysis for both gavage and inhalation animals.

APPENDIX G
Protocol No. 0ENT-24-11-07-03
Acute Inhalation Toxicity and Blood Absorption of DNAN in Rats

Individual Urine Concentrations For Multi-Time Point Blood Absorption Test

Inhalation

Animal #	Sex	Exposure Level	DNAN Urine Concentration (ug/ml)	2,4-DNP Urine Concentration (ug/ml)
12-0069	Male	.856 mg/L	7.7	130
12-0070	Male	.883 mg/L	1.3	0
12-0071	Male	.904 mg/L	15.0	5.2
12-0076	Female	.873 mg/L	5.8	0
12-0077	Female	.890 mg/L	23.0	9
12-0078	Female	.901 mg/L	4.4	0
		Mean	**9.53**	**24.03**
		S.D.	**8.033**	**52.043**

Oral Gavage

Animal #	Sex	Equivalent Oral Dose	DNAN Urine Concentration (ug/ml)	2,4-DNP Urine Concentration (ug/ml)
12-0072	Male	112.4 mg/kg	ND	ND
12-0073	Male	113.2 mg/kg	1.9	73
12-0074	Male	117.8 mg/kg	0.36	2.1
12-0079	Female	157.4 mg/kg	ND	ND
12-0080	Female	144.5 mg/kg	ND	ND
12-0081	Female	156.6 mg/kg	ND	ND
		Mean	**1.13**	**37.55**
		S.D.	**1.089**	**50.134**

ND = No data. Animal died on study.

Appendix H

Body Weights

APPENDIX H
Protocol No. 0ENT-24-11-07-03
Acute Inhalation Toxicity and Blood Absorption of DNAN in Rats

Individual Body Weights (grams)

Acute LC_{50} Test (0.7 mg/L)

Sex	Animal ID	Day 1	Day 2	Day 3	Day 6	Day 8	Day 13	Day 15
	12-0006	239	239	255	272	287	324	330
Male	12-0009	225	228	241	275	299	340	364
	12-0010	226	241	244	284	296	351	372
	12-0012	238	234	249	282	302	342	355
	12-0014	243	244	257	284	302	336	352
	Mean	**234.2**	**237.2**	**249.2**	**279.4**	**297.2**	**338.6**	**354.6**
	SD	**8.17**	**6.30**	**6.87**	**5.55**	**6.22**	**9.84**	**15.84**
	12-0017	183	188	196	216	218	249	256
Female	12-0020	182	180	185	205	211	231	225
	12-0022	184	181	190	204	218	230	236
	12-0024	182	184	195	202	206	219	216
	12-0026	186	186	187	208	214	229	232
	Mean	**183.4**	**183.8**	**190.6**	**207.0**	**213.4**	**231.6**	**233.0**
	SD	**1.67**	**3.35**	**4.83**	**5.48**	**5.08**	**10.85**	**14.93**

Acute LC_{50} Test (2.4 mg/L)

Sex	Animal ID	Day 1	Day 2	Day 3	Day 7	Day 10	Day 15
	12-0041	247	249	262	297	323	355
Male	12-0042	253	250	265	309	336	375
	12-0044	243	239	252	285	312	345
	12-0045	258	261	278	314	342	378
	12-0046	263	263	277	321	349	390
	Mean	**252.8**	**252.4**	**266.8**	**305.2**	**332.4**	**368.6**
	SD	**8.07**	**9.79**	**10.89**	**14.29**	**14.88**	**18.23**
	12-0049	192	186	190	195	204	212
Female	12-0051	206	204	212	222	223	238
	12-0052	211	209	218	230	240	251
	12-0053	206	210	208	223	236	243
	12-0054	195	185	193	207	207	224
	Mean	**202.0**	**198.8**	**204.2**	**215.4**	**222.0**	**233.6**
	SD	**8.09**	**12.36**	**12.17**	**14.15**	**16.36**	**15.57**

Multi-Timepoint Blood Absorption Test

Sex	Exposure Method	Animal ID	Day 1	Day 2
	Inhalation	12-0069	276	264
Male	Inhalation	12-0070	259	256
	Inhalation	12-0071	265	241
		Mean	**266.7**	**253.7**
		SD	**8.62**	**11.68**
	Gavage	12-0072	283	ND
Male	Gavage	12-0073	281	265
	Gavage	12-0074	270	246
		Mean	**278.0**	**255.5**
		SD	**7.00**	**13.44**
	Inhalation	12-0076	197	201
Female	Inhalation	12-0077	209	204
	Inhalation	12-0078	192	189
		Mean	**199.3**	**198.0**
		SD	**8.74**	**7.94**
	Gavage	12-0079	202	ND
Female	Gavage	12-0080	220	ND
	Gavage	12-0081	203	ND
		Mean	**208.3**	
		SD	**10.12**	

ND = No data. Animal died on study.

Appendix I

Clinical Observations

APPENDIX I
Protocol No. 0ENT-24-11-07-03
Acute Inhalation Toxicity and Blood Absorption of DNAN in Rats

Individual Clinical Signs

Acute LC_{50} Test (0.7 mg/L)

Sex	Animal ID	Observation	First Day Observed	Last Day Observed
Male	12-0006	Yellow-Stained Fur on Face/Head	1	15
		Salivation	1	1
		Red-Colored Discharge Around Nose	1	1
		Terminal Sacrifice 11/30/11		
	12-0009	Yellow-Stained Fur on Face/Head	1	15
		Salivation	1	1
		Red-Colored Discharge Around Nose	1	1
		Terminal Sacrifice 11/30/11		
	12-0010	Yellow-Stained Fur on Face/Head	1	15
		Salivation	1	1
		Red-Colored Discharge Around Nose	1	1
		Terminal Sacrifice 11/30/11		
	12-0012	Yellow-Stained Fur on Face/Head	1	15
		Salivation	1	1
		Terminal Sacrifice 11/30/11		
	12-0014	Yellow-Stained Fur on Face/Head	1	15
		Salivation	1	1
		Red-Colored Discharge Around Nose	1	1
		Terminal Sacrifice 11/30/11		
Female	12-0017	Yellow-Stained Fur on Face/Head	1	15
		Salivation	1	1
		Terminal Sacrifice 11/30/11		
	12-0020	Yellow-Stained Fur on Face/Head	1	15
		Salivation	1	1
		Red-Colored Discharge Around Nose	1	1
		Terminal Sacrifice 11/30/11		
	12-0022	Yellow-Stained Fur on Face/Head	1	15
		Salivation	1	1
		Red-Colored Discharge Around Nose	1	1
		Terminal Sacrifice 11/30/11		
	12-0024	Yellow-Stained Fur on Face/Head	1	15
		Salivation	1	1
		Red-Colored Discharge Around Nose	1	1
		Terminal Sacrifice 11/30/11		
	12-0026	Yellow-Stained Fur on Face/Head	1	15
		Salivation	1	1
		Red-Colored Discharge Around Nose	1	1
		Terminal Sacrifice 11/30/11		

Acute LC_{50} Test (2.4 mg/L)

Sex	Animal ID	Observation	First Day Observed	Last Day Observed
Male	12-0041	Yellow-Stained Fur on Face/Head	1	15
		Salivation	1	1
		Terminal Sacrifice 12/13/11		
	12-0042	Yellow-Stained Fur on Face/Head	1	15
		Salivation	1	1
		Terminal Sacrifice 12/13/11		
	12-0044	Yellow-Stained Fur on Face/Head	1	15
		Salivation	1	1
		Red-Colored Discharge Around Nose	1	1
		Terminal Sacrifice 12/13/11		
	12-0045	Yellow-Stained Fur on Face/Head	1	15
		Salivation	1	1
		Red-Colored Discharge Around Nose	1	1
		Terminal Sacrifice 12/13/11		
	12-0046	Yellow-Stained Fur on Face/Head	1	15
		Salivation	1	1
		Terminal Sacrifice 12/13/11		
Female	12-0049	Yellow-Stained Fur on Face/Head	1	15
		Salivation	1	1
		Terminal Sacrifice 12/13/11		
	12-0051	Yellow-Stained Fur on Face/Head	1	15
		Salivation	1	1
		Red-Colored Discharge Around Nose	1	1
		Terminal Sacrifice 12/13/11		
	12-0052	Yellow-Stained Fur on Face/Head	1	15
		Salivation	1	1
		Terminal Sacrifice 12/13/11		
	12-0053	Yellow-Stained Fur on Face/Head	1	15
		Salivation	1	1
		Red-Colored Discharge Around Nose	2	2
		Terminal Sacrifice 12/13/11		
	12-0054	Yellow-Stained Fur on Face/Head	1	15
		Salivation	1	1
		Terminal Sacrifice 12/13/11		

Appendix J

Necropsy Findings

APPENDIX J
Protocol No. 0ENT-24-11-07-03
Acute Inhalation Toxicity and Blood Absorption of DNAN in Rats

Gross Pathological Observations

Acute LC_{50} Test (0.7 mg/L)

Sex	Animal ID	Gross Observations
Male	12-0006	Staining on head, chin, and forearms Mildly enlarged spleen
	12-0009	Minimal staining of hair, mouth, jaw, and forearm areas
	12-0010	Minimal staining on head, chin, and forearms.
	12-0012	Staining on head, chin, and forearms
	12-0014	Minimal staining on head, nose, chin, and forearms
Female	12-0017	Minimal staining around chin, nose, and right forearm
	12-0020	Minimal staining of hair, mouth, jaw, and forearm areas
	12-0022	Minimal staining on head and chin
	12-0024	Staining on chin and right forearm
	12-0026	Moderate staining of head, neck, chest, and forearms

Acute LC_{50} Test (2.4 mg/L)

Sex	Animal ID	Gross Observations
Male	12-0041	Minimal yellow staining on chin
	12-0042	Yellow staining on chin and head Mottled lungs Froth in lower trachea
	12-0044	Mild staining on head Slightly enlarged spleen
	12-0045	Minimal yellow staining on head, nose, and chin
	12-0046	Minimal amount of yellow staining around nose and chin
Female	12-0049	Yellow staining on chin and forearms
	12-0051	Yellow staining on head, chest, and forearms
	12-0052	Small amount of yellow staining on chest, forearms, chin, and nose
	12-0053	Yellow staining of head, forearms, and chin
	12-0054	Minimal yellow staining of head, chin, and forearms

Appendix K

Study Protocol With Modifications

ANIMAL USE PROTOCOL
TOXICOLOGY PORTFOLIO
ARMY INSTITUTE OF PUBLIC HEALTH
U.S. ARMY PUBLIC HEALTH COMMAND
ABERDEEN PROVING GROUND, MD 21010-5403

PROTOCOL TITLE: Acute Inhalation Toxicity and Blood Absorption of 2,4-Dinitroanisole (DNAN) in Rats

PROTOCOL NUMBER: ØENT-24-11-Ø7-Ø3

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I. NON-TECHNICAL SYNOPSIS

The acute toxicity of 2,4-Dinitroanisole (DNAN), an insensitive, energetic material used in explosive formulations, will be evaluated by conducting a series of three experiments/tests. The first test will determine the acute inhalation toxicity (LC_{50}) of the test substance. Groups of 10 rats each will be exposed to a single, 4-hour atmospheric concentration of the test substance in air. An evaluation of the acute inhalation toxicity data will determine the relationship, if any, between the exposure of the rats to the test substance and the incidence and severity of abnormalities, including behavioral and clinical abnormalities, the reversibility of observed abnormalities, gross lesions, body weight changes, effects on mortality, and any other toxic effects. The second test, conducted in conjunction with the acute inhalation test, will serve as a rangefinding tool, and will collect a single, terminal blood sample from a small number of rats following dosage of the test substance by either the inhalation or oral gavage route of administration. The data generated from the rangefinding study will be used to select appropriate dosage concentrations for the multi-timepoint blood sample test. The third test will collect blood samples at multiple timepoints from 12 catheterized rats to determine the effect that two different routes of administration (inhalation and oral) have

on the absorption of the chemical into the blood. All rats will be monitored throughout the study for body weights and clinical signs. The acute inhalation test rats will be euthanized and receive a gross necropsy following the 14-day recovery period. Rats from the rangefinding test will be anesthetized and have blood samples collected prior to euthanasia. Rats from the multi-timepoint blood absorption test will be euthanized following collection of the final blood sample. Regulatory test guidelines typically state that the rat is the preferred species for this type of study. Historically, rats have been used for acute inhalation and oral toxicity studies and therefore are the recommended species due to the extensive historical database.

II. BACKGROUND

II.1. Background: 2,4-Dinitroanisole (DNAN) is being investigated as a less sensitive direct replacement for traditional explosives such as TNT and RDX. DNAN is a wax-like solid material that is one of the components used in the formulation of an insensitive munitions explosive referred to as IMX101. DNAN is used industrially in the synthesis of dyes and has been used as an insecticide in the past by the US Military. The use of DNAN as an energetic material in explosive formulations dates back to World War II when it was used as the main ingredient in Amatol 40 for various warheads. At the time, DNAN's use as an ingredient in explosive formulations was based primarily on the scarcity of higher performance materials, such as TNT. Renewed interest in the energetic properties of DNAN has been fueled by the need to develop munitions that are less prone to inadvertent initiation during transport and routine handling. The reduced sensitivity to environmental stimuli and nearly equal performance during testing make DNAN-based formulations desirable replacements for currently fielded munitions (reference 1). As a potential new component of munitions formulations, DNAN must not only meet certain performance criteria, but must also be acceptable from the perspective of human health and the environment. To ensure its safe use by military personnel and production employees handling the material on a daily basis, the toxicity and metabolism of DNAN must be investigated. Toxicological testing will be conducted by the U.S. Army Public Health Command (Provisional) (USAPHC (Prov)), Portfolio of Toxicology (TOX).

II.2. Literature Search for Duplication

II.2.1. Literature Source(s) Searched: AGRICOLA, BRD, DTIC, FEDRIP, NTIS, TOXLINE, PubMed, Web of Science

II.2.2. Date of Search: 09 March 2011

II.2.3. Period of Search: 1900-2011

II.2.4. Key Words of Search: 2,4-dinitroanisole, anisoles, dnan, aerosol, inhalation, breath, lung, nose, pulmonary, respiration, toxicity, blood, concentration, rats, mice, mouse, rodent, guinea pigs, animals

II.2.5. Results of Search: A total of 63 references resulted from the literature search that was performed for DNAN. However, no inhalation toxicity studies or blood

absorption studies for DNAN were found that would suggest that this study would be a duplicate effort. References related to acute inhalation toxicity data for DNAN were also found in a publication by the Australian Government Department of Defense (reference 1). In this publication, the acute inhalation toxicity of DNAN vapor was reported to be greater than 3 mg/m^3 and for an aerosol atmosphere of DNAN mixed with acetone the acute toxicity was reported to be greater than 2000 mg/m^3. Neither of these studies is considered to be an adequate representation of the expected inhalation exposure scenario for DNAN because they were either conducted at an insufficient concentration (3 mg/m^3 DNAN vapor) or were conducted with DNAN being mixed with a solvent (acetone). As such, the present study is not a duplication of the information available in the literature.

III. OBJECTIVE/HYPOTHESIS

The primary objective of this study is to determine the acute inhalation toxicity of DNAN. Rats will be exposed to atmospheres of DNAN and an estimate of the inhalation median lethal concentration (LC_{50}) will be determined. The LC_{50} is defined as the calculated atmospheric concentration of test substance expected to cause death in 50% of exposed animals either on the day of exposure or within at least 14 days post exposure. The secondary objective of this study will be to determine the effect that two different routes of administration (inhalation and oral) have on the absorption of the chemical into the blood.

IV. MILITARY RELEVANCE

As a result of an initiative by the Department of Defense (DOD) to improve munitions safety, the US Army is developing insensitive munitions (IM) for incorporation into its inventory of conventional military munitions systems. The Army's IM Program is dedicated to developing munitions that reliably perform as they are intended but are less prone to inadvertent initiation from external stimuli such as bullet/fragment impact, heat from fire, and shock from neighboring explosions (reference 2). The production of insensitive munitions requires the use of intrinsically less sensitive explosives that contribute to lower order responses to inadvertent external stimuli. Despite the slightly lower performance of DNAN compared to TNT, there has been a renewed interest in DNAN use in explosive formulations based on its lower sensitivity as a melt-cast medium observed during testing and the less stringent shipping requirements. This has led to the development of a range of melt-castable explosives at Picatinny Arsenal, collectively known as "PAX" explosives (reference 1). To support possible fielding of these PAX explosives, a Toxicity Clearance would have to be granted and occupational exposure guidelines developed. Consequently, toxicity data in a mammalian system need to be generated to assess occupational health hazards associated with the use and production of this material.

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures: This study consists of three experiments in which the toxicity of DNAN will be evaluated in rats: an acute inhalation toxicity test (LC_{50}), a rangefinding blood absorption test, and a multi-timepoint blood

absorption test. For the acute inhalation toxicity test, group(s) of 5 male and 5 female rats will be exposed (nose-only) for a single, 4-hour period to an atmosphere of the test substance in air. Multiple inhalation exposures may be conducted (each with a different group of rats) in order to determine the LC_{50}. Following the recovery period, surviving rats will receive a gross necropsy. For the inhalation phase of a rangefinding blood absorption test, groups of 2 rats each will be exposed by inhalation to different concentrations of DNAN (single exposure; nose-only, one male and one female), and following exposure, rats will be anesthetized (isoflurane or CO_2 gas) and a single terminal blood sample (approximately 3-6 ml) will be collected via cardiac puncture. Immediately following blood collection, rats will be euthanized by CO_2 (euthanasia will be ensured by pneumothorax). An additional component of the rangefinding blood absorption test will consist of groups of 2 rats each being dosed orally (single dose; gavage; one male and one female). Following the oral dose, all rats will be anesthetized (isoflurane or CO_2 gas) and a single terminal blood sample (approximately 6 ml) will be collected via cardiac puncture. Immediately following blood collection, rats will be euthanized by CO_2 (euthanasia will be ensured by pneumothorax). For the multi-timepoint blood absorption test, one group of 12 rats, each fitted with a subcutaneous femoral artery catheter, will either be exposed to DNAN by inhalation (single exposure; nose-only; 3 male and 3 female) or dosed orally (single dose; gavage; 3 male and 3 female). The absorption of DNAN will be determined from blood samples collected from each rat via the arterial catheter at up to 7 selected timepoints during the test. Immediately following the final blood collection sample, each rat will be euthanized by injection of a solution of sodium pentobarbital into the catheter and euthanasia will be ensured by pneumothorax. All rats will be monitored throughout the study for mortality/moribundity, body weights, and/or clinical signs. Estimated initiation date for the study is July 2011. Estimated completion date for the study is September 2011.

V.1.1. Experiment 1: Acute Inhalation Toxicity (LC_{50}) Test

In an attempt to determine the acute toxicity associated with inhalation exposure to DNAN, groups of 10 rats each (5 male and 5 female) will be exposed nose-only to a single, 4-hour atmosphere of the test substance in air. Following exposure, surviving rats will be retained for at least a 14-day recovery period. In the event that significant signs of toxicity (e.g., mortality, neurotoxicity, etc.) are delayed, the duration of the recovery period may be extended in order to determine the length of time for recovery, however, the recovery period will not exceed 28 days. During the test, rats will be monitored for morbidity/mortality, weight loss, and/or clinical signs of toxicity. Rats that survive the recovery period will be euthanized by CO_2 (euthanasia will be ensured by pneumothorax) and then necropsied. All rats will receive a gross necropsy. Generally for acute inhalation toxicity studies, if no mortality occurs in exposed animals at a limit concentration (2000 mg/m^3) of the test substance being tested, only one exposure is required. However, since this acute inhalation test with DNAN is not expected to be a limit test, multiple exposures with additional groups of animals are expected to be conducted following the initial exposure. These subsequent exposures will be conducted at concentrations appropriately adjusted based on the results of previous exposure(s). Up to a total of 5 exposures may need to be conducted in order to determine the LC_{50} for DNAN. If one sex is observed to be more sensitive to the toxicological properties of the test substance, subsequent exposures with 10 rats of the

more sensitive sex may be conducted. The primary endpoint of this study is mortality attributed to exposure of the test substance. The number of rats per group that expire either during the exposure or during the recovery period constitutes the fractional mortality for each exposure. In an attempt to minimize potential issues related to the shipment of rats that are not within the desired age or weight ranges, one additional rat per sex will be ordered for each exposure to ensure that each exposure is initiated with 5 male and 5 female rats within the proper age and weight ranges. A total of 12 rats will be ordered for each exposure with a total of 10 rats actually exposed. A maximum of 5 exposures will be conducted. Each of the "additional" animals not used for the inhalation exposure will be used for the rangefinding work to be conducted in Experiment 2. However, if for some reason the decision is made not to use these "additional" animals for the rangefinding work, they will be transferred to another protocol or humanely euthanized per protocol guidance. Details of the experimental design and general procedures for an acute inhalation toxicity study are described in TOX SOP 029 (reference 3), with modifications and clarifications detailed in this protocol.

Experiment 1. Acute Inhalation Toxicity (LC_{50}) Test

Exposure No. / Design Concentration	No. of Male Rats (a)	No. of Female Rats (a)
1. 500-1000 mg/m^3	5 + 1 = 6	5 + 1 = 6
2. TBD	5 + 1 = 6	5 + 1 = 6
3. TBD	5 + 1 = 6	5 + 1 = 6
4. TBD	5 + 1 = 6	5 + 1 = 6
5. TBD	5 + 1 = 6	5 + 1 = 6
	TOTAL = 30 (a)	**TOTAL = 30 (a)**

(a) Five rats/sex will be used for each exposure. One additional rat/sex will be ordered for weight matching purposes. Each of the rats designated as "additional" for Experiment 1 will be used for the rangefinding test in Experiment 2 (see below).

V.1.2. Experiment 2: Rangefinding Blood Absorption Test

A rangefinding blood absorption test will be conducted to determine the effect that different inhalation concentrations of DNAN and the route of dose administration has on the absorption of DNAN into the blood of rats. The data generated from the rangefinding test will be used to select appropriate dosage concentrations for the multi-timepoint blood absorption test (Experiment 3). The effect that varying the concentrations of DNAN has on the absorption of DNAN into the blood of rats will be examined by exposing groups of 2 rats each (one male and one female) to different concentrations of DNAN atmospheres. For each inhalation exposure, the 2 rats designated as "additional" from the acute inhalation exposure (Experiment 1) will be exposed nose-only to a single, 4-hour atmosphere of the test substance concurrently with the 10 rats being exposed in the acute inhalation exposure (Experiment 1). Following the exposure (e.g., within 30 minutes), these 2 rats will be anesthetized (isoflurane or CO_2 gas) and a single blood sample (approximately 3-6 ml) will be collected via cardiac puncture. The effect that the route of administration has on the absorption of DNAN into the blood of rats will be examined by orally dosing (gavage) one group of rats (one male and one female) to a selected concentration of DNAN at a dose considered similar to one of the inhalation exposure concentrations conducted during the acute inhalation test (Experiment 1). Following administration of the dose

(e.g., within 1 hr), these 2 orally dosed rats will be anesthetized (isoflurane or CO_2 gas) and a single blood sample (approximately 3-6 ml) will be collected via cardiac puncture. Immediately following blood collection, all rats will be euthanized by CO_2 and euthanasia will be ensured by pneumothorax. All blood samples will be collected and evaluated per TOX SOP 053 (reference 4). Blood samples will immediately be injected into a vessel containing a measured volume of solvent specified by Army Institute of Public Health (AIPH) Laboratory Science (LS) personnel. LS personnel will determine the most appropriate analytical method for analyzing the concentration of DNAN or the metabolite 2,4-dinitrophenol in blood. The details of the analytical method will be documented in the study records and final report. Two rats will be used for each inhalation exposure and these rats will be obtained from the "additional" rats ordered from the acute inhalation test (Experiment 1) for the purpose of weight matching. A maximum of 5 exposures will be conducted, so a maximum of 10 rats will be used for inhalation administration during Experiment 2. Additionally, 2 rats will be used for each oral gavage dose group. These rats will be ordered specifically for the oral dose administration phase of the test in which 2 rats will be receive an oral gavage dose of DNAN. A maximum of 5 dose groups will be conducted (to coincide with the maximum number of inhalation exposures), so a maximum of 10 rats will be used for the oral administration during Experiment 2. Any animals not used for this rangefinding test will be transferred to another protocol or humanely euthanized per protocol guidance.

Experiment 2. Rangefinding Blood Absorption Test

Exposure Route / Exposure or Dose No. / Design Concentration	No. of Male Rats	No. of Female Rats
Inhalation / EXP#1 / TBD	1 (a)	1 (a)
Inhalation / EXP#2 / TBD	1 (a)	1 (a)
Inhalation / EXP#3 / TBD	1 (a)	1 (a)
Inhalation / EXP#4 / TBD	1 (a)	1 (a)
Inhalation / EXP#5 / TBD	1 (a)	1 (a)
Oral / DOSE#1 / TBD	1 (b)	1 (b)
Oral / DOSE#2 / TBD	1 (b)	1 (b)
Oral / DOSE#3 / TBD	1 (b)	1 (b)
Oral / DOSE#4 / TBD	1 (b)	1 (b)
Oral / DOSE#5 / TBD	1 (b)	1 (b)
	TOTAL = 5 (a,b)	**TOTAL = 5 (a,b)**

(a) Rats will be obtained from the 2 additional rats ordered for each of the acute inhalation (LC50) exposures (Experiment 1); therefore, these rats are already accounted for in the table for Experiment 1 (see above) and are not included in the TOTAL listed here; the only rats included in the TOTAL here are the rats ordered specifically for the oral dosing phase of this rangefinding test.

(b) Two rats (one male and one female) will be ordered specifically for the oral dosing phase of this rangefinding test.

V.1.3. Experiment 3: Multi-Timepoint Blood Absorption Test

A multi-timepoint blood absorption test will be conducted to determine the absorption rate of DNAN into the blood of rats following the administration of the test substance by two different routes of administration (inhalation and oral gavage). Rats will be ordered from the vendor with matching ages/weights and each rat will be received with a subcutaneous femoral artery catheter in place. One group of 6 rats (3 male and 3 female) will be exposed nose-only to a single, 4-hour atmosphere of the test substance in air and another group of 6 rats (3 male and 3 female) will receive a single, oral

gavage dose of the test substance. The absorption of DNAN will be determined from blood samples collected from each rat at selected timepoints during the test. A single blood sample (approximately 0.15 ml) will be drawn from each rat at selected timepoints. For the rats exposed by inhalation, blood samples will be drawn at each of 7 timepoints : (1) approximately 1-2 hours prior to initiation of the inhalation exposure, (2) approximately 1 hour from initiation of inhalation exposure, (3) approximately 2 hours from initiation of inhalation exposure, (4) immediately following (e.g., within 30 minutes) the conclusion of the 4-hr inhalation exposure (5) approximately 4 hours following the conclusion of the inhalation exposure, (6) approximately 8 hours following the conclusion of the inhalation exposure, and (7) following an overnight recovery period (approximately 18-hours). For the rats dosed by oral gavage, blood samples will be drawn at each of 6 timepoints : (1) approximately 1-2 hours prior to the dose, (2) approximately 1 hour following the dose, (3) approximately 2 hours following the dose, (4) approximately 4 hours following the dose, (5) approximately 8 hours following the dose, and (6) following an overnight recovery period (approximately 18-hours).The blood sample collected at each timepoint will not exceed 0.2 ml and the total blood volume collected during the 24-hour blood collection period will not exceed 7.5% of the circulatory blood volume for the rats (reference 5). Blood samples will be collected from the subcutaneous femoral artery catheter of each rat utilizing the following process:

- To draw a blood sample from the catheter, one of the study personnel will gently restrain the animal while a second individual performs the actual blood collection procedure.
- The catheter plug, together with the catheter, will be pulled 1-2 inches caudally out of the skin pocket.
- While holding the junction of the plug and the polyurethane tubing with forceps, a hemostat or a second set of forceps will be used to remove the plug.
- A 1 cc syringe fitted with a 23 gauge luer stub needle adaptor will be inserted into the catheter tubing and the lumen lock solution (heparinized solution) will be withdrawn.
- The catheter will then be crimped with a cushioned hemostat.
- A clean 1 cc syringe fitted with a 23 gauge luer stub needle adaptor will be inserted into the catheter and the hemostat will be removed in order to withdraw the blood sample.
- Approximately 0.15 ml of blood will be drawn from each rat at each sample timepoint for whole blood analysis.
- After the blood samples are withdrawn, the catheter will be crimped with a hemostat and a saline-filled syringe will be inserted into the catheter.
- The catheter will be flushed with approximately 0.2 ml of saline solution and crimped again with a hemostat.
- A syringe filled with heparinized saline solution will be inserted into the catheter and the dead volume of the catheter will be filled with heparinized saline solution and plugged in order to prevent clotting.
- The catheter may be wiped with an alcohol swab if necessary, inserted back into the skin flap, and secured in place with a wound clip (reference 6).

Blood samples will immediately be injected into a vessel containing a measured volume of solvent specified by AIPH Laboratory Science (LS) personnel. LS personnel will determine the most appropriate analytical method for analyzing the concentration of DNAN or the metabolite 2,4-dinitrophenol in blood. The details of the analytical method will be documented in the study records and final report. Following the final blood collection sample, each rat will be euthanized by injection of a solution of sodium pentobarbital into the catheter and euthanasia will be ensured by pneumothorax. A total of 14 rats will be needed to conduct this blood absorption test. One additional rat/sex is being ordered to ensure that if there are any problems with the catheter there are at least 6 rats/sex/administration route remaining to place on test. Since it is not critical to have similar body weights for the rats being tested, no attempt will be made to perform any type of computerized, randomization program for grouping. Animals not used for this blood absorption test will not be transferred to another protocol due to the subcutaneous femoral artery catheter, and therefore, will be humanely euthanized per protocol guidance.

Experiment 3. Multi-Timepoint Blood Absorption Test

Exposure Route / Design Concentration	No. of Male Rats	No. of Female Rats
Inhalation / TBD	3	3
Oral / TBD	3	3
Additional Rats Ordered for Potential Catheter Clogging Problems	1	1
	TOTAL = 7 (a)	**TOTAL = 7 (a)**

(a) Six rats (3/sex) will be used for each route of exposure. One additional rat/sex will be ordered for weight matching purposes. However, since 2 of the rats will be obtained from the 2 additional rats ordered for one of the acute inhalation (LC50) exposures (Experiment 1), these rats are already accounted for in the table for Experiment 1 (see above). The other 2 rats will be ordered specifically for this rangefinding test.

V.1.4. Test Substance: This study will be conducted with 2,4-Dinitroanisole (DNAN). The sample was supplied by BAE SYSTEMS, Ordnance Systems, Kingsport, TN for use in a previously conducted subchronic oral toxicity study in rats. The test sample was received at the test facility in August 2010 and is identified as Batch# 10DNAN9-9 and Lot# BAE10H281-008. Following is a list of relevant information concerning the chemical/physical properties of the test substance.

Test Substance Chemical/Physical Properties

Name	2,4-Dinitroanisole
Synonym	DNAN
CAS#	119-27-7
Physical State	Tan powder (wax-like)
Molecular Formula	$C_7H_6N_2O_5$
Molecular Weight	198
Density	1.34 g/cm^3
Melting Point	94-96 ºC
Solubility	Practically insoluble in water
Purity	>99% (Approx)

V.1.5. Administration of Test Substance by Inhalation: Rats being evaluated for inhalation toxicity and blood absorption of DNAN via inhalation administration will be exposed nose-only to airborne concentrations of the test substance. The nose-only

(head-only) exposure mode is typically used for test atmospheres that contain particulates/aerosols in an attempt to minimize deposition of the test substance onto the fur of the animals, and therefore, minimizing inadvertent dermal and oral exposure of the test substance to the animals. Since the generation method of DNAN for this study is expected to produce a test atmosphere containing a mix of both aerosol and vapor components, the nose-only exposure mode is considered to be the most appropriate mode. Rats will be individually restrained during exposure in perforated, stainless steel cylinders with conical nose pieces. This type cylinder design is typically used for nose-only inhalation exposures and is widely accepted for inhalation toxicity test systems (references 7 and 8). Rats will be positioned in the exposure cylinders such that their noses will be at the conical end of the cylinder. In order to secure the rat in this position, a plastic disk with a hole in the center will be inserted over the tail of each rat and positioned within the cylinder near the base of the rat's tail to prevent the rat from backing out of the rear of the cylinder. Care will be taken to properly insert each rat into its exposure cylinder, such that there is a balance between allowing the rat adequate space to move while ensuring that it is positioned properly for adequate exposure. Each exposure cylinder will be inserted into one of the holes in the faceplate of the exposure chamber such that only the nose/head of each rat extends into the exposure chamber. Animal nose-only exposure cylinders and related equipment will be appropriately cleaned after each use.

V.1.6. Administration of Test Substance by Oral Gavage Dose: Rats being evaluated for blood absorption of DNAN via oral administration will be orally dosed (gavaged) using a stainless steel 16 ga x 2 inch gavage needle. As per EPA Health Effects Test Guidelines, the volume given will not exceed 10 ml/kilogram of body weight (reference 9). Due to the acute nature of the blood absorption tests, no attempts will be made to analyze the purity/concentration of the DNAN solutions to be dosed.

V.1.7. Concentration Selection for Inhalation Exposure(s): For the acute inhalation toxicity test (LC_{50}), the design concentration for the initial exposure will be based on the acute oral toxicity classification of DNAN. The acute oral toxicity classification for DNAN was determined in a previous study (reference 1) in which DNAN was classified as having moderate acute oral toxicity (LD_{50} = 199 mg/kg). Referring to the EPA Toxicity Categories (reference 10), the concentration listed for a test substance categorized as moderately toxic in an inhalation acute test (LC_{50}) ranges from 0.2 to 2 mg/L. If the assumption is made that the acute inhalation toxicity classification for DNAN will be similar to the acute oral toxicity classification, the concentration range of 0.2 to 2 mg/L seems to be a reasonable range to use for the initial exposure. For practical purposes, the overall range will likely be narrowed to 500-1000 mg/m^3 as the design concentration for the initial exposure. The design concentrations for subsequent exposures will be appropriately adjusted based on the results of previous exposure(s). For the blood absorption tests, the design concentration for the inhalation exposures will be based on results of the acute inhalation test and/or any rangefinding work conducted during this study in conjunction with analytical guidance from AIPH Laboratory Sciences (LS) personnel to ensure appropriate limits of detection for blood analysis.

V.1.8. Dose Selection for Oral Gavage: Dose selection for the rats administered oral doses as part of the blood absorption tests will be based on the acute oral toxicity

classification of DNAN. The acute oral toxicity classification for DNAN was determined in a previous study (reference 1) in which DNAN was classified as having moderate acute oral toxicity (LD_{50} = 199 mg/kg). Referring to the EPA Toxicity Categories (reference 10), the concentration range listed for a test substance categorized as moderately toxic by acute oral testing ranges from 50 to 500 mg/kg. Therefore, the oral dose administered to rats on the blood absorption tests will likely range from 50 to 500 mg/kg. The actual dose selection will be based on results of method development work conducted during this study in conjunction with analytical guidance from AIPH Laboratory Sciences (LS) personnel to ensure appropriate limits of detection for blood analysis.

V.1.9. Inhalation Exposure Duration: For inhalation administration, each group of rats will be exposed for 4 hours to the test atmosphere. For the acute inhalation toxicity test and the rangefinding blood absorption test, the starting time of the exposure will be defined as the time when the generation system is turned on and the ending time of the exposure will be defined as the time when the generation system is turned off. Rats will be exposed to the test substance during both the time it takes for the chamber to reach concentration, and the time it takes for the test substance to be purged from the chamber. For the multi-timepoint blood absorption test, the rats will be loaded into the exposure system chamber in staggered increments (e.g., approximately 15-minute increments) in order to accommodate the blood collection schedule. The time that each rat is loaded into the exposure system will be recorded in the study records and will represent the beginning of the exposure period for that individual rat. Rats will be removed from the exposure chamber approximately one and two hours after the start of the exposure so that blood samples can be collected. The time that each rat is removed from the chamber, the time of the blood collection, and the time that each rat is returned to the exposure chamber will be recorded in the study records. The time that rats are not in the exposure chamber during the blood collections will be made up at the end of the exposure to ensure that each rat receives a 4-hour exposure. At the end of each exposure, all rats will be removed from the exposure cylinders.

V.1.10. Inhalation Atmosphere Generation: Attempts will be made to generate atmospheres of the DNAN in the exposure chamber which can be readily respired by the rats being exposed and which approximate the physical form of the test substance expected to be encountered in real-life situations. Chamber atmospheres will be generated dynamically, and attempts will be made to produce evenly distributed mixtures of the test substance in air with a minimum airflow of at least 10 air changes per hour in the exposure chamber. Measurements will be taken during the method development phase and the generation system altered, if needed, to strive for uniform distribution of the test substance within the breathing zone of the exposed animals. The methods of chamber distribution are described in TOX SOP 152 (reference 11). Test atmospheres will be generated by heating the test substance, and therefore, due to expected condensation effects, the test atmosphere in the exposure chamber is expected to contain an aerosol component in addition to the vapor component. For the aerosol component, attempts will be made to generate respirable-sized particles (e.g., mass median aerodynamic ranges from 1 to 4µm) that would be expected to be deposited throughout the respiratory tract. If the targeted particle size cannot be attained, the most respirable atmosphere practically attainable will be tested. The

interior atmosphere of the exposure chamber will be slightly negative in relation to its surroundings. Test atmospheres may be exhausted through appropriate exhaust equipment (e.g., scrubbers, HEPA filters) prior to discharge into ventilated exhaust piping. The actual generation equipment and experimental conditions used will be documented in the study records and described in the final report.

V.1.11. Analysis of the Test Atmosphere: A suitable analytical method, approved by the study director, will be used to determine the atmospheric concentration (and particle size) of the test substance in the general breathing zone of the exposed rats. The method of aerodynamic particle size measurement is described in TOX SOP 041 (reference 12). Since the chamber atmosphere is expected to contain both vapor and aerosol components, the analytical method will need to accurately quantify both components. Chamber analysis samples will typically be collected by TOX study personnel, however, some analyses may need to be performed by AIPH Laboratory Sciences (LS) personnel. Unless prohibited by the nature of the test substance, chamber environmental conditions (e.g., airflow, temperature, humidity, etc.) will be monitored continuously and collected at least 3 times during each exposure. All exposure chamber analytical and environmental data will be documented in the study records. Details of the actual analytical methods and equipment used will be documented in the study records and described in the final report.

V.1.12. Grouping of Study Animals: Animals that have been released from quarantine, have no overt signs of disease, and are of the appropriate sex, age, and body weight range will be randomly selected for the study. The weight variation in animals used for the acute inhalation test exposures should not exceed ± 20% of the mean weight of each sex.

V.1.13. Animal Body Weights and Observations: All rats will be weighed at least once per week during the exposure/recovery period. Body weights will be collected (at a minimum) just prior to exposure/dosing, the day following exposure/dosing, and the final day of recovery. Additionally, rats will be weighed on the day following each instance of weight loss attributed to toxicity of the test material. Rats typically will not be weighed on weekends or holidays unless warranted by their health status. Individual body weights of animals will be documented in the study records by study personnel.

A thorough physical examination of each rat will be performed by study personnel at least once per day (weekends and holidays excluded unless warranted by health status). The examination process will consist of each rat being removed from its home cage, individually handled, and carefully observed. Observations will include, but not be limited to, evaluation of skin and fur, eyes and mucous membranes, respiratory and circulatory effects, autonomic effects such as salivation, central nervous system effects, including tremors and convulsions, changes in the level of activity, gait and posture, reactivity to handling or sensory stimuli, altered strength, and stereotypes or bizarre behavior (e.g., self mutilation, walking backwards). In addition, rats exposed via the inhalation route of administration will be observed at least 3 times while they are in the exposure chamber (unless restricted by the characteristics of the test atmosphere or exposure system) and will also be thoroughly observed immediately following their removal from the exposure system. Subsequent observations of rats up to several

hours following the exposure may also be conducted by study personnel if warranted by health status of the rats. All data related to the observation of rats will be detailed and thoroughly documented in the study records by study personnel. In addition, a brief summary related to the collection of body weights and observations will also be recorded in the animal room logbook on days that this data is collected. If any animals die during the exposure or recovery periods, the day and time of death will be recorded as precisely as possible. Rats will also be observed by Veterinary Medicine staff during the acclimation, exposure, and recovery periods (including weekends and holidays).

V.1.14. Gross Pathology: A gross necropsy will be performed on all animals tested as part of the acute inhalation toxicity test (Experiment 1). Following the 14-day recovery period, all surviving rats will be euthanized by carbon dioxide and undergo a gross necropsy. Based on findings during the gross necropsy, some tissues may be saved for future histopatholgical examination. The decision to save tissues will be documented in the study records and the results will be included in the final report. All data related to the necropsy will be recorded on CHPPM Form 333. If the necropsy cannot be performed immediately after the death of an animal, the animal will be refrigerated at temperatures low enough to minimize autolysis.

V.1.15. Study Conduct: This study will be conducted in a manner consistent with the principles of 40 CFR (Code of Federal Regulations) Part 792 "Toxic Substance Control Act" (TSCA) Good Laboratory Practice (GLP) Regulation (reference 13). All study records will be made available to oversight organizations such as the Environmental Protection Agency or the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) as needed. The investigators and technicians will adhere to The Guide for Care and Use of Laboratory Animals, 2011 (reference 14).

Records will be kept in standard USAPHC laboratory notebooks and/or three ring binders. Daily records will be kept on survival and clinical signs collected on the animals during the exposure and recovery periods. Procedures for preparation of any euthanasia solution, drug administration, animal bleeds, observation logs, morbidity/mortality logs, etc., will be stored with the study records. These records will be made available to oversight organizations such as the US EPA, AAALAC, and the IACUC. The protocol, protocol amendments, raw data, statistical analysis, tabular calculations, and graphic analysis of the data will be saved with the study records. Additionally, memoranda to the study file, study logs, signature logs, final reports, and final report amendments will be archived at USAPHC. Some ancillary records such as maintenance and calibration logs, environmental monitoring logs, animal room log books, all veterinarian staff duties logbooks, training files, etc. may be stored in the archives but not stored with the study files.

V.2. Data Analysis: For the acute inhalation toxicity test, if the test substance fails the limit concentration test, then an LC_{50} value will be estimated using a probit analysis (reference 15) and 95% confidence limits estimated if possible. In the absence of confidence limits, an approximate LC_{50} may be reported. For the multi-timepoint blood absorption test, a comparison of the concentration of DNAN in the blood samples across times will be performed with a randomized block analysis of variance (ANOVA) followed by a Tukey's or Dunnett's C test to compare pairs of times. The use of 6

animals per dose administration is considered adequate for determination of a 50% or greater difference in test material blood concentrations between groups at alpha equal 5% with power of 80%. Statistical significance for all tests is defined as $p<.05$. Descriptive statistics (e.g., mean, standard deviation, and standard error of the mean) will be used to summarize experimental data (e.g., atmospheric concentrations). If additional statistics are required, they will be documented in the study records and included in the final report.

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered: The objective(s) addressed by this study are adverse health effects observed in rats administered the test substance by inhalation and oral administration. The data from this study will aid in the assessment and evaluation of the toxic characteristics of the test substance. There are no appropriate animal substitutes (e.g., computer models, tissue/cell cultures) for the data that will be produced in this study. No non-animal alternative would provide the necessary toxicological information provided by this study. Therefore, it is necessary to perform this study in an animal model.

V.3.2. Animal Model and Species Justification: The test guidelines for the U.S. Environmental Protection Agency (EPA) and Organisation for Economic Co-Operation and Development (OECD) state that the rat is the preferred species (references 16 and 17). Sprague-Dawley rats are the strain of rat that have been historically used for acute inhalation and oral toxicity studies by USAPHC TOX and are the recommended species due to an historical and extensive database.

V.3.3. Laboratory animals

V.3.3.1. Genus and Species: *Rattus norvegicus*

V.3.3.2. Strain/Stock: Sprague-Dawley

V.3.3.3. Source vendor: Charles River Laboratories, Wilmington, MA (USDA 14-R-0144) or other USAPHC approved vendor

V.3.3.4. Age (at exposure): Approximately 8-10 weeks

V.3.3.5. Weight (at exposure): Age appropriate; exposed rats all within ± 20% of group mean for their exposure group

V.3.3.6. Sex: Male and female. Only one sex (the more sensitive sex) will be used when obvious sex differences relative to toxicity are noted.

V.3.3.7. Special Considerations: None

V.3.4. Number of Animals Required (By Species):

Minimum of 28 rats (if only one acute inhalation toxicity exposure is conducted)

NUMBER OF RATS IF MINIMUM NUMBER OF EXPOSURES CONDUCTED

Type Test	Concentration	# Rats Ordered	# Rats Exposed/Dosed	# Additional Rats
Acute Inhalation (LC_{50})	500-1000 mg/m^3	12	10	2
Rangefinding Blood Absorption	TBD	2	4	(a)
Multi-Timepoint Blood Absorption	TBD	14 (b)	12	2
TOTAL		**28**	**26**	**2 (a)**

(a) Two rats (one/sex) will be used for each route of exposure (total of 4 rats). Two of the rats will be obtained from the 2 additional rats ordered for the acute inhalation (LC50) exposure and the other 2 rats will be ordered specifically for this rangefinding test.
(b) Each rat will be received from the animal supplier with a subcutaneous femoral artery catheter in place

Maximum of 84 rats (if a total of 5 acute inhalation toxicity exposures are conducted)

NUMBER OF RATS IF MAXIMUM NUMBER OF EXPOSURES CONDUCTED

Type Test / Exposure No.	Concentration	# Rats Ordered	# Rats Exposed/Dosed	# Additional Rats
Acute Inhalation (LC_{50}) / Exp#1	500-1000 mg/m^3	12	10 *[Pain Category C]*	2
Acute Inhalation (LC_{50}) / Exp#2	TBD	12	10 *[Pain Category C]*	2
Acute Inhalation (LC_{50}) / Exp#3	TBD	12	10 *[Pain Category C]*	2
Acute Inhalation (LC_{50}) / Exp#4	TBD	12	10 *[Pain Category E]*	2
Acute Inhalation (LC_{50}) / Exp#5	TBD	12	10 *[Pain Category E]*	2
Rangefinding Blood Absorption	TBD	10 (a)	20 (a) *[12 rats @ Pain Cat D] [8 rats @ Pain Cat E]*	0 (a)
Multi-Timepoint Blood Absorption	TBD	14 (b)	12 (b) *[Pain Category C]*	2 (b) *[Pain Category B]*
TOTAL		**84**	**82**	**2 (a)**

(a) Two rats (one/sex) will be used for each inhalation exposure (total of 10 rats); these 10 the rats will be obtained from the 2 additional rats ordered for each of the acute inhalation (LC50) exposures; two rats (one/sex will be used for each oral gavage dose (total of 10 rats); these 10 rats will be ordered specifically for the rangefinding test.
(b) Each rat will be received from the animal supplier with a subcutaneous femoral artery catheter in place

For the acute inhalation toxicity test (LC_{50}), at least one group of 10 rats will be exposed to a concentration of 500-1000 mg/m^3 DNAN, however, depending on the results of this exposure and any subsequent exposures, as many as 4 additional exposures may need to be conducted to accurately determine the inhalation median lethal concentration

(LC_{50}) for DNAN. Twelve rats (6 male and 6 female) will be ordered for each of the acute inhalation toxicity (LC_{50}) exposures, with 10 rats (5 male and 5 female) being exposed during each exposure and one rat per sex designated as "additional". A minimum of 5 rats per sex for each of the acute inhalation toxicity (LC_{50}) exposures is required by the EPA and OECD Health Effects Test Guidelines (reference 16 and 17). "Additional" rats need to be ordered for weight matching purposes because these same guidelines require that the body weight variation for each exposure group of rats prior to exposure be within ± 20% of the mean weight of each sex. The "additional" animals (one female rat and one male rat) from Experiment 1 will be used for the inhalation exposure phase of the rangefinding test (Experiment 2).

Although there are no specific regulatory guidelines for the blood absorption tests to be conducted as part of this study, the number of rats being used for these tests is considered to be appropriate. For the multi-timepoint blood absorption test, a total of 7 rats/sex will be received for each dose administration (inhalation and oral), with 6 rats (3 male and 3 female) being exposed/dosed and one rat being designated as "additional". The use of 6 animals per dose administration is considered adequate for determination of a 50% or greater difference in test material blood concentrations between the two different dose administrations at alpha equal 5% with power of 80%. For the rangefinding blood absorption test, groups of 2 rats (one rat/sex) for each of the dose administrations (inhalation and oral) is considered to be the minimum number of rats required to provide adequate rangefinding results.

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V.3.5. Refinement, Reduction, Replacement

V.3.5.1. Refinement: Standard rat enrichment will be implemented in accordance with TOX SOP 122 (reference 18). For rats assigned to the acute inhalation test and the rangefinding blood absorption test, rats may be pair-housed (sexes separate) during the acclimatization period. However, during the conduct of the study, rats from these tests will need to be single-housed due to the unknown toxicity of the test substance. For rats assigned to the multi-timepoint blood absorption test, rats will need to be singly housed upon their arrival and throughout the test due to the subcutaneous femoral artery catheter that each of the rats will have in place upon arrival. All animals on this study will be handled on a frequent basis and provided a form of environmental enrichment (e.g., nylabones) throughout the study period, except during the 4-hour exposure period. Another refinement is that moribund animals or animals in overt pain and unlikely to recover will be humanely euthanized.

V.3.5.2. Reduction: Ten rats (e.g., 5 male and 5 female) per exposure is the number of animals specified by the applicable regulatory guidelines for an acute inhalation toxicity study (references 16 and 17). Although there are no specific regulatory guidelines for blood absorption tests being conducted as part of this study, the number of animals being requested is in the range of generally accepted number per sex for standard acute inhalation/oral toxicity studies. No control groups will be used. Tissue sharing may be allowed (except for rats with subcutaneous femoral artery catheter), however, only if doing so will not affect the validity of the study.

V.3.5.3. Replacement: No non-animal alternatives are known to exist that will provide the required data. At this time, there are no non-animal alternatives that can fully replicate the complex processes that occur within an intact mammalian organism.

V.4. Technical Methods

V.4.1. Pain/Distress Assessment:

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals

NOTE: Estimates listed in Columns B-E below are modeled after a maximum number of 5 exposures conducted for the acute inhalation toxicity test.

V.4.1.1.1.1. Column B: 2 rats (2 "additional" rats ordered with subcutaneous femoral artery catheter for use on the multi-timepoint blood absorption test)

V.4.1.1.1.2. Column C: 42 rats (10 rats per exposure at 3 lower concentrations conducted for the acute inhalation test; 12 rats ordered with subcutaneous femoral artery catheters to be exposed/dosed as part of the multi-timepoint blood absorption test)

V.4.1.1.1.3. Column D: 12 rats (2 rats per exposure at the 3 lower concentrations for the inhalation phase of the rangefinding test; 2 rats per dosing concentration at the 3 lower concentrations for the oral dosing phase of the rangefinding test)

V.4.1.1.1.4. Column E: 28 rats (10 rats per exposure at 2 higher concentrations conducted for the acute inhalation test; 2 rats per exposure at the 2 higher concentrations for the inhalation phase of the rangefinding test; 2 rats per dosing concentration at the 2 higher concentrations for the oral dosing phase of the rangefinding test)

V.4.1.2. Pain Relief/Prevention

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization: For the rats assigned to the rangefinding blood absorption test, anesthesia will be administered prior to cardiac blood collection and euthanasia. Anesthesia will consist of isoflurane or CO_2 gas. For isoflurane anesthesia, study staff will ensure the oxygen tank and isoflurane levels are sufficiently full and scavenger canisters are connected to both exhaust lines. The stopcock to the box will be turned to the open position and the stopcock to the nosecone to the off position. The oxygen tank will be turned on, the flow meter set to 1 L/min, the rat placed in the plastic box, and the lock latched. The isoflurane valve will be turned to approximately 3%. Once the rat is sufficiently anesthetized (immobile and not responsive to tapping on the box), the stopcock to the nosecone will be switched to on and the stopcock to the box to off. The rat will be transferred to the nosecone and it will be ensured that the rat is still sufficiently anesthetized, based on lack of responsiveness to toe-pinch, before performing terminal blood sampling. For CO_2 anesthesia, study staff will ensure that the CO_2 tank is sufficiently full and connected to

the CO_2 chamber. The rat will be placed in the CO_2 chamber, the lid put on the chamber, and the CO_2 valve turned on at a low flow (approx, ¼ turn on the tank valve). When the rat is sufficiently anesthetized (shallow breathing pattern) it will be removed from the chamber and immediately, quickly placed on a necropsy board, where prior to performing the terminal blood sampling, sufficient anesthetization will be ensured by the rat's lack of responsiveness to a toe-pinch.

V.4.1.2.2. Pre- and Post-procedural Provisions: Animals will be monitored just prior to exposure/dosing, during exposures, and immediately following exposure (while being returned to their cages) and/or dosing. A careful clinical examination will be made at least once each day during the observation period. Appropriate actions will be taken to minimize loss of animals or associated relevant data to the study (e.g., necropsy or refrigeration of those animals found dead). Observations will be detailed and carefully recorded in the study records. Observations will include, but not be limited to, evaluation of skin and fur, eyes and mucous membranes, respiratory and circulatory effects, autonomic effects such as salivation, central nervous system effects, including tremors and convulsions, changes in the level of activity, gait and posture, reactivity to handling or sensory stimuli, altered strength, and stereotypes or bizarre behavior (e.g., self mutilation, walking backwards). Observation and body weight frequency is described in detail in section V.2.12.

V.4.1.2.3. Paralytics: None

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Source(s) Searched: AGRICOLA, FEDRIP, NTIS

V.4.1.3.2. Date of Search: 09 March 2011

V.4.1.3.3. Period of Search: 1964-2011

V.4.1.3.4. Key Words of Search: 2,4-dinitroanisole, anisoles, dnan, aerosol, inhalation, breath, lung, nose, pulmonary, respiration, toxicity, blood, concentration, alternative, welfare, method, model, in vitro, pain, distress, simulate, video, computer, replacement, refinement, reduction

V.4.1.3.5. Results of Search: The literature search did not identify any references pertaining to alternatives to painful or distressful procedures. There are no alternatives to the painful/distressful procedures (e.g., illness due to administration of the test material, cardiac bleed) in this protocol or methods to relieve pain or distress without altering the outcome of the study were found. Since the goal of this investigation is to determine the effects from acute inhalation and/or oral exposure (e.g., lethality from a one-time administration of the test substance) the observation of illness associated with toxicity is necessary. However, moribund animals or animals in overt pain unlikely to recover will be humanely euthanized as described in section V.4.6. Because no validated in vitro tests are currently available to replace in vivo inhalation/oral toxicity studies, this protocol must be conducted in vivo.

V.4.1.4. Unalleviated Painful/Distressful Procedure Justification: The nature of this type of study precludes the use of totally painless procedures. Since the objective of this study is to determine the toxicological effects from acute inhalation exposure and/or acute oral dosage to the test substance, no materials that could potentially interfere or mask the interpretation of these toxicological effects will be administered to the study animals. The primary endpoint of the acute inhalation test is death, therefore, it is important that the investigators are confident in the outcome before pain or distress can be alleviated. Since animals will receive a single dose of the test substance, subsequent recovery may occur. Therefore, pain and/or distress will be alleviated only if it is judged that animals are unlikely to recover. Administration of anesthetics, analgesics, or drugs in this model to alleviate pain or distress is untested and may alter the manifestation of the toxic response to the compound, and thus compromise the results of the experiment. Typical pain relievers such as opiates and non-steroidal anti-inflammatories as well as anesthetics have the ability to mask certain toxic signs that may be observed due to the administration of the test compound, especially those signs resulting from pain or distress. In addition, the observation of the onset, duration and/or reversibility of toxic signs is critical to mechanistic interpretation; “Toxic signs” are defined in TOX SOP 063 (reference 19). However, the Attending Veterinarian will be consulted to evaluate animals that appear moribund and the Attending Veterinarian and Primary Investigator/Study Director (PI/SD) will determine if euthanasia is indicated for these animals. One or more of the clinical signs will be considered to be indicative of a moribund animal: impaired ambulation which prevents animals from reaching food/water; excessive weight loss or emaciation (≥ 20% body weight loss from start of test); lack of physical or mental alertness; prolonged labored breathing; unabated seizure activity; inability to urinate or defecate for greater than 24 hours; or a prolonged inability to remain upright. Animals considered to be moribund will be euthanized as described in section V.4.6. The final number of rats in each pain category will be reported to the IACUC annually and at the completion of the in-life portion of the protocol.

V.4.2. Prolonged Restraint: For nose-only exposures, rats will be restrained in perforated, stainless steel cylinders during the 4-hour exposure period. This type of restrainer and the restraint regimen is a commonly accepted method of restraint for rats used during nose-only inhalation exposures (references 7 and 8). Rats will be contained within the nose-only cylinders longer than the 4-hour exposure period due to the additional time required to load the rats into the cylinders prior to the exposure and then unloading them from the cylinders following the exposure. The time period that the rats are actually contained within the exposure cylinder, allowing for the loading/unloading process, however, will not exceed 5 hours. Current IACUC policy defines prolonged restraint as any restraint greater in duration than 15 minutes and requires that restrained animals be habituated or undergo acclimation to the restraint device in the event of prolonged restraint unless scientifically justified to be unnecessary (reference 20). Acclimation of the rats to the nose-only exposure cylinders is not considered to be scientifically justified for this study because it is a single exposure and the primary endpoint is mortality. If the endpoints for this study included collection of critical parameters such as sensitive blood chemistries or physiological measures, or if it was a repeated-dose study evaluating endpoints such as body weights, acclimation of the rats to the exposure cylinders would probably be considered scientifically justified.

However, since this is a single-dose study, it is not considered necessary to acclimate the animals on this study to the nose-only exposure cylinders prior to exposure. Furthermore, the exposure cylinders are not considered to be stressful to the rats in and of themselves since their design is similar to the enrichment tubes typically placed in the rats' cages. The only stressful situation for rats restrained in these exposure cylinders is considered to be the first few minutes of exposure to the test atmosphere, and an acclimation period to the exposure cylinder prior to the actual exposure would not prevent this type of stress.

V.4.3. Surgery: None

V.4.3.1. Pre-Surgical Provisions: N/A

V.4.3.2. Procedure: N/A

V.4.3.3. Post-Surgical Provisions: N/A

V.4.3.4. Location: N/A

V.4.3.5. Surgeon: N/A

V.4.3.6. Multiple Major Survival Operative Procedures: None

V.4.3.6.1. Procedures: N/A

V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations

V.4.4.1. Injections: None

V.4.4.2. Biosamples: For the rats assigned to the rangefinding blood absorption test, approximately 3-6 ml of blood will be taken from each rat just prior to euthanasia. All blood sampling will occur under isoflurane or CO_2 gas anesthesia via cardiac puncture using an 18-21 gauge, 1-1.5 inch needle, as outlined in TOX SOP 053 (reference 4). Biosampling will be promptly followed by euthanasia via CO_2. For the 12 rats assigned to the multi-timepoint blood absorption test, a single blood sample (approximately 0.15 ml) will be drawn from the subcutaneous femoral artery catheter of each rat at each of up to 7 timepoints (see section V.2.3 for details). The blood sample collected at each timepoint will not exceed 0.2 ml and the total blood volume collected during the 24-hour blood collection period will not exceed 7.5% of the circulatory blood volume for the rats (reference 5). Following the final blood collection sample, each rat will be euthanized by injection of approximately 1 ml of a solution of sodium pentobarbital into the catheter or by exposure to CO_2.

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification: Animals will be identified by cage cards according to TOX SOP 003 (reference 21). An identification number (e.g., the last 3 digits of the animal number) will also be marked on the tail of each rat with a water-insoluble marker in order to ensure proper identification of rats when removed from their cages or inhalation exposure system.

V.4.4.6. Behavioral Studies: N/A

V.4.4.7. Other Procedures: N/A

V.4.4.8. Tissue Sharing: Tissue sharing may be allowed upon request provided there is no affect on the validity of the study. Rats fitted with a subcutaneous femoral artery catheter will not be available for tissue sharing.

V.4.5. Study Endpoint: The study endpoint is mortality, intervention euthanasia of moribund animals, or euthanasia. The duration of the recovery period will not typically exceed 14 days. In the event that significant signs of toxicity (e.g., mortality, neurotoxicity, etc.) are delayed, the duration of the recovery period may be extended in order to determine the length of time for recovery, however, the recovery period will not exceed 28 days. The possibility exists that a compound-related death may occur during an unobserved period (i.e., overnight). Intervention euthanasia will be conducted on moribund animals. Animals will be assessed for moribundity based on a weight of evidence of the following signs: impaired ambulation which prevents animals from reaching food/water; excessive weight loss or emaciation (≥ 20% body weight loss from start of test); lack of physical or mental alertness; prolonged labored breathing; unabated seizure activity; inability to urinate or defecate for greater than 24 hours; or a prolonged inability to remain upright. Any animal considered moribund will be humanely euthanized as described in section V.4.6. The Attending Veterinarian will be consulted, if needed, to evaluate potentially moribund animals, unless the PI/SD plans to immediately euthanize the animal. The time at which signs of toxicity appear, their duration, and the time to death are important, especially if there is a tendency for deaths or morbidity to be delayed or if the signs of toxicity are reversible or recovery is possible. As such, potentially moribund animals will be monitored, in consultation with the Attending Veterinarian, for possible reversal and recovery of toxic signs.

At the end of the recovery period, all surviving animals assigned to the acute inhalation test and the rangefinding blood absorption test will be euthanized by CO_2 ensured by pneumothorax. Rats assigned to the multi-timepoint blood absorption test, which will be fitted with subcutaneous femoral artery catheters, will be euthanized by injection of a sodium pentobarbital-based solution into the catheter.

V.4.6. Euthanasia: Euthanasia for the rats assigned to the acute inhalation toxicity test and the rangefinding blood absorption test will be accomplished by asphyxiation from CO_2 exposure according to TOX SOP 066 (reference 22). Rats assigned to the multi-timepoint blood absorption test may be euthanized by injection of a solution of sodium pentobarbital (approximately 1 ml) into their subcutaneous femoral artery catheter (however, rats may also be euthanized by CO_2 exposure). Death of all rats will be ensured with a thoracotomy. Study staff will euthanize the animals.

V.5. Veterinary Care

V.5.1. Husbandry Considerations: The animals will be housed in plastic, solid-bottom shoebox cages and given water and certified rodent feed *ad libitum* during the study. For rats assigned to the acute inhalation test and the rangefinding blood absorption test, rats may be pair-housed (sexes separate) during the acclimatization period. However, during the conduct of the study, rats from these tests will need to be single-housed due to the unknown toxicity of the test substance. For rats assigned to the multi-timepoint blood absorption test, rats will need to be singly housed upon their arrival and throughout the test due to the subcutaneous femoral artery catheter that each of these rats will have in place upon arrival. Animal rooms will be maintained according to the conditions specified in TOX SOP 004 (reference 23). For rats assigned to the acute inhalation test and the rangefinding blood absorption test, animals will undergo an acclimation period of no less than 5 days after their arrival in the animal facility. Rats assigned to the multi-timepoint blood absorption test will not have the standard 4- to 5-day acclimation period. Instead, these rats will be exposed/dosed the day following arrival at the testing facility to minimize the potential for clogging problems with the subcutaneous femoral artery catheter that could occur with a longer acclimatization period.

V.5.1.1. Study Room: Studies will be conducted at the USAPHC Toxicology Portfolio animal facility, Bldg E-2100 or Bldg E-2101, study room as assigned. The animal facilities are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

V.5.1.2. Special Husbandry Provisions: Rats assigned to the acute inhalation test and the rangefinding blood absorption test may be pair-housed (sexes separate) during the acclimatization period. However, during the conduct of the study, rats from these tests will need to be single-housed due to the unknown toxicity of the test substance. For rats assigned to the multi-timepoint blood absorption test, due to the subcutaneous femoral artery catheter that each of the rats will have in place, these rats will need to be singly housed upon their arrival and throughout the test.

V.5.1.3. Exceptions: For the acute inhalation toxicity test and the rangefinding blood absorption test, body weight and observation data may also be collected for rats by study personnel during the acclimation period in an attempt to more accurately monitor the health status of the rats in preparation for their use on study. However, animals will not be weighed or handled by study personnel within the first 24 hours after their arrival to the facility. For the multi-timepoint blood absorption test, since these rats will be fitted with subcutaneous femoral artery catheters, the catheters may be flushed by study personnel on the day of their arrival in an attempt to minimize plugging of the catheter.

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care: All animals will be observed daily by assigned Veterinary Medicine personnel for husbandry conditions, humane care, and general health. Animals will be observed at least twice daily by assigned Veterinary Medicine personnel (once daily on weekends and holidays). During the exposure

period, animals exposed by inhalation will be observed by study personnel prior to loading them into the exposure chamber, during the exposure, and then again after they are removed from the exposure chamber. In addition, during the recovery period, study animals will be observed at least once daily (weekends and holidays excluded) by study personnel. Observations will include, but not be limited to: evaluation of skin and fur, eyes and mucous membranes, respiratory and circulatory effects, autonomic effects such as salivation, central nervous system effects, including tremors and convulsions, changes in the level of activity, gait and posture, reactivity to handling or sensory stimuli, altered strength, and stereotypes or bizarre behavior (e.g., self mutilation, walking backwards). If there is a need for increased frequency of observations, the duty veterinarian will consult with the PI/SD. Appropriate actions will be taken to minimize loss of animals to the study (e.g., necropsy or refrigeration of those animals found dead). If the observed toxicity indicates a need for more frequent observations, the Attending Veterinarian will consult with the PI/SD.

All rats will be weighed at least once per week during the exposure/recovery period. Body weights will be collected (at a minimum) just prior to exposure/dosing, the day following exposure/dosing, and the final day of recovery. Additionally, rats will be weighed on the day following each instance of weight loss attributed to toxicity of the test material. Rats typically will not be weighed on weekends or holidays unless warranted by their health status.

Observation and body weight data collected by study personnel will be documented in the study records. A brief summary related to the collection of body weights and observations will also be recorded in the animal room logbook on days that this data is collected.

V.5.2.2. Emergency Veterinary Medical Care: All emergency animal health care will be provided by the Veterinary Medicine staff in consultation with the PI or designee whenever possible.

V.5.3. Environmental Enrichment

V.5.3.1 Enrichment Strategy: All enrichment will be provided in accordance with TOX SOP 122 (reference 18). Animals will be handled on a frequent basis and provided a form of environmental enrichment (e.g., nylabones) throughout the study, except during the 4-hour exposure period.

V.5.3.2. Enrichment Restriction: For rats assigned to the acute inhalation test and the rangefinding blood absorption test, rats may be pair-housed (sexes separate) during the acclimatization period. However, during the conduct of the study, rats from these tests will need to be single-housed due to the unknown toxicity of the test substance. For rats assigned to the multi-timepoint blood absorption test, due to the subcutaneous femoral artery catheter that each of the rats will have in place, these rats will need to be singly housed upon their arrival and throughout the test.

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

Staff Member	Procedure	Training	Experience	Qualifications
Art O'Neill	Inhalation exposure, observations, handling, CO_2 euthanasia, necropsy	Inhalation testing experience (memo from DuPont dated Oct 2008); necropsy (Dec 2007)	30+ Yrs Animal Research	B.S., Biology; LATG
Lee Crouse	Inhalation exposure, oral gavage, observations, handling, bleeding, CO_2 euthanasia, anesthesia, necropsy	Humane Care & Use of Lab Animals (May 2000); Rodent Handling Techniques, WRAIR (includes oral gavage in rats; Nov 1996); Rat handling, gavage, injections, blood collection (July 2007); Rat cardiac bleeding under isoflurane (Dec 2008, May 2009); necropsy (Oct/Dec 2007)	16+ Yrs Animal Research	M.S., Environmental Science
Emily Lent	Oral gavage, observations, handling, bleeding, CO_2 euthanasia, Necropsy	Rat handling, gavage, injections, blood collection (July 2007); Rat bleeding techniques & tissue collection (Apr 2008); necropsy (Jul/Oct 2007, Apr 2008); Rat oral gavage (March 2008); Oral gavage in rats (May 2009)	11+ Yrs Animal Research	M.S., Wildlife Biology; Ph.D., Natural Resources and Environmental Studies
Mark Way	Observations, handling, CO_2 euthanasia, Necropsy	Rodent & Small Animal Handling workshops (2003, 2007); necropsy (May 2007)	17+ Yrs Animal Research	B.S., Biology; LAT
Terry Hanna	Observations, handling, CO_2 euthanasia, necropsy, functional observation battery (FOB)	Rodent Handling & Techniques (1992); Rodent & Small Animal Handling Workshop (2004, 2005, 2006); Rat handling and gavage (2007), rat euthanasia via CO_2 with thoracotomy (3/2009); rat isoflurane anesthesia, cardiac blood draw, & CO_2 euthanasia (2009); necropsy (2009, 2010); Functional observation battery (FOB) training (5/2007, 8/2008, 1/2009); Acoustic Startle Response (handheld clicker & startle chamber operations) (1/2009)	15+ Yrs Animal Research	ALAT

Will McCain	Observations, handling, necropsy	Animal Care & Use Training (Mar 1995); Humane Care & Use of Lab Animals (May 2000); necropsy (Dec 2007, Feb/Dec 2008, Feb 2009)	30+ Yrs Animal Research	Ph.D., Toxicology
Alicia Shiflett	Observations, handling, necropsy	Rodent handling & techniques training; observations, handling/restraint, weighing, basic bleeding (Nov 2008); rat CO_2 euthanasia with thoracotomy (Mar 2009); rat necropsy & tissue collection (Mar 2008, Jan 2010)	2+ Yrs Animal Research	Associates Degree, Histology/Science

VII. BIOHAZARD/SAFETY:

In accordance with PHC Reg. 385-1, CHPPM Reg. 385-5, and TOX SOP 083, standard laboratory protection (e.g., glasses, gloves, labcoat) shall be used when handling the neat test substance. The test substance shall be stored in a sealed container at room temperature when not in use. The test substance will be handled in a laboratory fume hood when necessary. Although the precise toxicity of the test substance may not be known, information regarding its chemical family is provided so that a reasonable assessment of its safety can be made (references 24, 25, and 26). Due to the potentially flammable/explosive properties of this material, equipment in the exposure system will be grounded.

VIII. ENCLOSURES:

A. References

IX. ASSURANCES:

IX.1. As the Study Director/ Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard/Safety: I have taken into consideration, and I have made the proper coordinations regarding all applicable rules and regulations regarding radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training : I verify that the personnel performing the animal procedures/manipulations/ observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures/manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical, and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedures: I am conducting biomedical experiments which may potentially cause more than momentary ~~or slight~~ pain or distress to animals. This potential pain and/or distress WILL or WILL NOT be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

Arthur J. O'Neill – Study Director (PI)

20110714
Date (YYYYMMDD)

IX.2. As the Primary Co-Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Authority: I understand that, as the Primary Co-Investigator, I am authorized and responsible for performing all procedures and manipulations as assigned to the SD/PI in the SD/PI's absence. This includes euthanasia of distressed animals.

C. Training: I verify that I am technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures/manipulations.

D. Responsibility: I acknowledge the inherent moral, ethical, and administrative obligations associated with the performance of this animal use protocol, and I assure that I will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

E. Painful Procedures: I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress WILL or WILL NOT be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

Lee C.B. Crouse – Primary Co-Investigator

20110714
Date (YYYYMMDD)

APPENDIX A

REFERENCES

1. Davies, P.J. and Provatas, A. Characterisation of 2,4-Dinitroanisole: An Ingredient for Use in Low Sensitivity Melt Cast Formulations. Defence Science and Technology Organisation, PO Box 1500, Edinburgh, South Australia 5111 Australia, DSTO-TR-1904, 2006.

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3. USAPHC DTOX SOP No. IT029-T-002, Acute Inhalation Toxicity Study, 2010.

4. USAPHC DTOX SOP No. AP053-002, Animal Bleeding Technique, 2011.

5. National Institute of Health, Guidelines for Survival Bleeding of Mice and Rats, http://oacu.od.nih.gov/ARAC/Bleeding.pdf, Revised 1/12/05.

6. Vascular Catheterizations: Handling Instructions. 2000. Charles River Laboratories, 251 Ballardvale Street, Wilmington, MA.

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9. Health Effects Test Guidelines. OPPTS 870.1100, Acute Oral Toxicity Study in Rodents. EPA 712–C–98–199, August 1998.

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19. USAPHC DTOX SOP No. AP063-002, Test System Observations, 2011.

20. USACHPPM IACUC Policy Memorandum Number 1, Guidelines on Prolonged Restraint, 20 February 2009.

21. USAPHC DTOX SOP No. AP003-P-001, Individual Animal Identification, 2009.

22. USAPHC DTOX SOP No. AP066-002, Animal Euthanasia, 2011.

23. USAPHC DTOX SOP No. AP004-P-001, Animal Health Technician Duties within the Animal Facility, 2009.

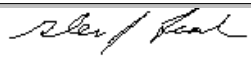
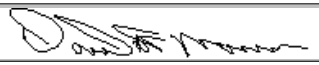
24. USAPHC Regulation 385-1, Safety and Occupational Health Program, 15 January 2010.

25. USACHPPM Regulation 385-5, Occupational Health and Safety of Animal Users, 1 June 2007.

26. USAPHC DTOX SOP No. GL083-P-002, Health and Safety of Laboratory Personnel, 2010.

PROTOCOL REVIEW, SUPPORT, APPROVAL SHEET

PROTOCOL NUMBER:	TITLE:
0ENT - 24 - 11-07-03 SUB-JONO TEST TYPE IACUC NUMBER	Acute Inhalation Toxicity and Blood Absorption of 2,4-Dinitroanisole (DNAN) in Rats

1. SCIENTIFIC MERIT (PEER REVIEW)			
1a. Printed Name (First, MI, Last) Craig A. McFarland	1b. Title Toxicologist, HERP	1c. Signature MCFARLAND.CRAIG.A.1284367789	1d. Date (yyyy/mm/dd) 20110526
2. DIRECTOR			
2a. Printed Name (First, MI, Last) LTC Cindy A. Landgren	2b. Title Director, Toxicology	2c. Signature LANDGREN.CINDY.ANNE.1163891359	2d. Date (yyyy/mm/dd) 20110526
3. PROGRAM MANAGER			
3a. Printed Name (First, MI, Last) Glenn J. Leach	3b. Title Program Manager, TEP	3c. Signature 	3d. Date (yyyy/mm/dd) 20110524
4. ATTENDING VETERINARIAN			
4a. Printed Name (First, MI, Last) MAJ Dawn C. Fitzhugh	4b. Title Command Animal Program Manager	4c. Signature FITZHUGH.DAWN.CATHERINE.1036926127	4d. Date (yyyy/mm/dd) 20110725
5. ANALYTICAL CHEMISTRY (If Applicable)			
5a. Printed Name (First, MI, Last) David F. Morrow	5b. Title Chief, Laboratory Consultants Division	5c. Signature 	5d. Date (yyyy/mm/dd) 20110526
6. SAFETY MANAGER			
6a. Printed Name (First, MI, Last) Roy A. Valiant	6b. Title Safety Manager	6c. Signature VALIANT.ROY.A.1081780591	6d. Date (yyyy/mm/dd) 20110718
7. STATISTICIAN (If Applicable)			
7a. Printed Name (First, MI, Last) Karen D. Deaver	7b. Title Statistician	7c. Signature DEAVER.KAREN.DEVILBISS.1400519672	7d. Date (yyyy/mm/dd) 20110526

PROTOCOL NUMBER:	TITLE: Acute Inhalation Toxicity and Blood Absorption of 2,4-Dinitroanisole (DNAN) in Rats		
0ENT - 24 - 11-07-03 SUB-JONO TEST TYPE IACUC NUMBER			
8. SIO-QAT (GLP COMPLIANCE AND QA SUPPORT)			
8a. Printed Name (First, MI, Last) Michael P. Kefauver	8b. Title Quality Assurance Specialist, USAPHC Quality Systems Office (QSO)	8c. Signature KEFAUVER.MICHAEL.P.1229209678	8d. Date (yyyy/mm/dd) 20110526
9. CHAIRMAN, IACUC			
9a. Printed Name (First, MI, Last) Kristin T. Newkirk	9b. Title Chairperson, IACUC	9c. Signature NEWKIRK.KRISTIN.TORELL.1014786895	9d. Date (yyyy/mm/dd) 20110727
10. INSTITUTIONAL OFFICIAL			
10a. Printed Name (First, MI, Last) John J. Resta	10b. Title Director, AIPH	10c. Signature RESTA.JOHN.J.1229129305	10d. Date (yyyy/mm/dd) 20110729
11. STUDY DIRECTOR/PRINCIPAL INVESTIGATOR			
11a. Printed Name (First, MI, Last) Arthur J. O'Neill	11b. Title Biologist, TEP	11c. Signature ONEILL.ARTHUR.J.III.1299508443	11d. Date (yyyy/mm/dd) 20110729
12. OTHER ORGANIZATION(S) PROVIDING SUPPORT (AS NEEDED):			
12a. Printed Name (First, MI, Last)	12b. Title	12c. Signature	12d. Date (yyyy/mm/dd)
13. STUDY SPONSOR:			
13a. Printed Name (First, MI, Last) Mark S. Johnson	13b. Title Program Manager, HERP (for RDECOM)	13c. Signature	13d. Date (yyyy/mm/dd)

USACHPPM PROTOCOL MODIFICATION

For use of this form, see IACUC SOP 1.0

1. DATE: (YYYY/MM/DD) 2011/10/31	2. PROTOCOL NUMBER: 0ENT-24-11-07-03	3. MODIFICATION#: 01

4. PROTOCOL TITLE: Acute Inhalation Toxicity and Blood Absorption of 2,4-Dinitroanisole (DNAN) in Rats

5. STUDY DIRECTOR/PRINCIPAL INVESTIGATOR:	6. WORK PHONE:	7. OFFICE SYMBOL:
Arthur J. O'Neill	410-436-5080	MCHB-IP-TTE

SECTION I. PREVIOUSLY APPROVED AND CURRENTLY IN USE PROTOCOL MODIFICATIONS:

1 MODIFICATION NUMBER	2. SHORT DESCRIPTION OF PRIOR APPROVED MODIFICATION(S)	3. NO. & SPECIES OF ANIMAL REQUESTED	4. APPROVED DATE

SECTION II. CHANGE IN TOTAL # OF ANIMALS USED AND/OR CHANGE IN USDA PAIN CATEGORY

1a. CHANGE: INCREASE TOTAL APPROVED ANIMALS BY: 0					1b. N/A ☒				
2. ORIGINAL PROTOCOL TOTAL: 84					3. PROTOCOL TOTAL AFTER MODIFICATION: 84				
2a. USDA pain cat:	B: 2	C: 42	D: 12	E. 28	3a. USDA pain cat:	B: 2	C: 42	D: 12	E: 28

4. Yes	No	
☒	☐	Modification requires specific changes or additions to the experimental design of the protocol. (Section V.I. of the template.)
☒	☐	Modification requires changes to the technical methods, i.e., procedures, routes of administration, biosample collection, etc. (Section V.4. of the protocol template.) Indicate training of personnel for new methods, procedures being used.
☐	☒	Modification requires additions or changes in personnel performing procedures. (Section VI of the protocol template.) Include training and qualification information and tasks that each individual will be performing. If changing the Study Director/PI, a signed Assurance Statement needs to be submitted with the modifications.

PROTOCOL Page, paragraph, section	**SECTION III. MODIFICATION/JUSTIFICATION** *Explain the modification indicated above in the area below. Indicate any changes to the 3R's (Refinement, Reduction, Replacement) resulting from changes in number of animals*
p. 6-8, V.1.3 p. 19, V.4.4.2	1. MODIFICATION: Urine samples will be collected from the 12 catheterized rats in Experiment 3 (Multi-Timepoint Blood Absoption Test). The 12 rats in Experiment 3 will be placed in a metabolism cage following their 8-hr bleed timepoint. Rats will remain in the metabolism cage overnight and urine samples will be collected. Rats will have food and water available while they are in the metabolism cage. Rats will not be housed in the metabolism cage longer than 12 hours.
	1a. JUSTIFICATION/REASON: The urine samples will be analyzed by LS personnel to determine the concentration of the test material and/or metabolites in the urine of rats exposed to DNAN. The purpose of this data is to determine if the urine of individuals working with IMX-101 can be used to monitor their expsoure to the test material.

PROTOCOL Page, paragraph, section	*Explain the modification indicated above in the area below. Indicate any changes to the 3R's (Refinement, Reduction, Replacement) resulting from changes in number of animals used.*
	2. MODIFICATION:
	2a. JUSTIFICATION/REASON:
	3. MODIFICATION:
	3a. JUSTIFICATION/REASON:
	4. MODIFICATION:
	4a. JUSTIFICATION/REASON:

Continued on next page YES ☐ NO ☒

SECTION IV. SIGNATURES AND DATES

1 STUDY DIRECTOR: (Printed Name) Arthur J. O'Neill		DATE: (yyyy/mm/dd) 2011/11/02
2. PROGRAM MANAGER:: (Printed Name) Dr. Glenn J. Leach		DATE: (yyyy/mm/dd) 2011/11/02
3. ATTENDING VETERINARIAN: (Printed Name) Dawn C. Fitzhugh, Maj, VC		DATE: (yyyy/mm/dd) 2011/11/03
4. CHPPM SAFETY OFFICER/OCC HEALTH REP: (IF APPLICABLE)		DATE: (yyyy/mm/dd)
5. CHAIR, IACUC: (Printed Name) APPROVED YES ☒ NO ☐ Kristin T. Newkirk		DATE: (yyyy/mm/dd) 2011/11/29