Bull Environ Contam Toxicol (2008) 80:487–491
DOI 10.1007/s00128-008-9467-9

Acute toxicity of 2,4,6-trinitrotoluene, 2,4-dinitrotoluene, and 2,6-dinitrotoluene in the adult bullfrog (*Lithobates catesbeiana*)

Norka E. Paden · Ernest E. Smith · Ronald J. Kendall

Received: 27 February 2007 / Accepted: 12 May 2008 / Published online: 13 June 2008

Abstract 2,4,6-Trinitrotoluene (TNT) is one of the most prevalent high explosives in the environment. 2,4-Dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT) are the most common isoforms of dinitrotoluene. The goal of this study was to determine the acute toxic effects of TNT, 2,4-DNT, and 2,6-DNT in adult male bullfrogs. The LD_{50} for TNT was 1,060 mg/kg BW while the LD_{50} for 2,4-DNT and 2,6-DNT was 1,098 mg/kg BW. All three compounds elicited similar symptoms of toxicity including changes of skin color, body weight, development of seizures, liver and kidney necrosis, and lung cyanosis. Relative organ weights did not show significant change.

Keywords Up-and-Down procedure · Bullfrog · LD_{50} · High explosives

Acute toxicity data constitute primary information to evaluate toxic characteristics of substances and to provide relevant information for environmental and hazard risk assessments (OECD 2001; Rispin et al. 2002). Recently, the U.S. EPA Up-and-Down (UPD) maximum likelihood test guideline was accepted as a replacement for traditional LD_{50} determination methods (Rispin et al. 2002). The advantage of this new methodology is the use of fewer animals for the determination of the median lethal dose (LD_{50}).

Oral acute toxicity using the UPD method of TNT, 2,4-DNT, and 2,6-DNT has been reported for several vertebrate species. Hispid cotton rats (*Sigmodon hispidus*) have been exposed to TNT (Reddy et al. 2000), effects of oral 2,4-DNT exposure to the Northern bobwhite (*Colinus virginianus*) have been investigated (Johnson 2005), and rats and mice have been exposed to 2,6-DNT (Lee et al. 1975; Ellis et al. 1978). However, little is known about the toxicity of explosive residues in amphibian species, particularly in the bullfrog *(Lithobates catesbeiana)*, a native American species. Thus, the objective of this study was to determine the LD_{50}s of the three compounds tested in the bullfrog.

Materials and Methods

2,4,6-TNT (CAS # 118-96-7), 2,4-DNT (CAS # 121-14-2), and 2,6-DNT (CAS # 606-20-20) were purchased from Chem Services and Alfa Aesar (Ward Hill, MA, USA) at 98%, 97%, and 97% purity, respectively. Polyethylene glycol (PEG) (CAS # 25322-63-3) from Sigma-Aldrich (St. Louis, MO, USA) was used as the carrier substance for all chemicals tested. Oral gavage stainless steel needles from Popper & Sons Inc. (New Hyde Park, NY, USA) were used to administer the suspension. Standards for TNT, 2,4-DNT, and 2,6-DNT (100% purity) were purchased from Accustandard (New Haven, CT, USA).

Dose suspensions were prepared approximately 22 h before the dosing and separately for each animal. Aliquots were diluted and measured in triplicate for each compound. Dilutions followed a three step procedure. First, a 1:100 dilution in acetonitrile was prepared. Second, a 1:100 dilution in water (50:50) was performed. The last step included a

N. E. Paden · E. E. Smith · R. J. Kendall
The Institute of Environmental and Human Health,
Texas Tech University, Lubbock, Texas 79409, USA

N. E. Paden (✉) · E. E. Smith · R. J. Kendall
Department of Environmental Toxicology,
Texas Tech University, Lubbock, Texas 79409, USA
e-mail: norka.paden@ttu.edu

1:2 dilution in acetonitrile (50:50). Diluted samples were measured using a Hewlett-Packard 1100 (Wilmington, DE, USA) reverse phase high performance liquid chromatography (HPLC) coupled with ultraviolet detection (wavelength: 254 nm) one hour prior to dosing. Water and acetonitrile (50:50) were used as the mobile phase which passed through a 25 cm × 4.6 mm × 5 µm Discovery® C18 column from Supelco (Bellefonte, PA, USA). Standards were prepared in acetonitrile by diluting to the desired concentration and stored in dark containers at 4°C. Suspensions of TNT, 2,4-DNT, and 2,6-DNT in PEG were administered to bullfrogs by oral gavage. Measured concentrations were considered acceptable if they were within 15% of the target dose (Smith 2007). The coefficient of variation of the three aliquot samples tested prior to dosing were 2.5%, 5.7%, and 6.8% for TNT, 2,4-DNT, and 2,6-DNT, respectively.

Water quality of the experiments was monitored regularly. Temperature, dissolved oxygen, pH, conductivity, and salinity, were checked daily using an YSI model 556 Multiprobe System (MPS) (Yellow Springs, OH, USA) for each tank. Ammonia was measured at the beginning and end of each experiment using a Nessler's N-NH3 (method 8038) in a calibrated Hach spectrophotometer model DR 2800 (Ames, IA, USA).

Twenty-four adult male bullfrogs (average SVL: 10.2 cm; average body weight: 180 g) were used. Frogs were purchased from Rana Ranch Bullfrog Farm (Twin Falls, ID, USA) and acclimatized for 2 weeks in an 888 L tank containing 118 L of medium. The medium consisted of carbon filtered, UV sterilized, reverse osmosis (RO), and de-ionized (DI) water supplemented with 0.33 mg/L Instant Ocean sea salts from Aquarium Systems (Mentor, OH, USA). Room temperature for both experiments was kept at 21 ± 2°C and photoperiod = 12:12 h light:dark. Three live crickets were fed to each animal three times a week; water changes were performed weekly. After acclimatization, animals in groups of three were transferred to 112 L glass tanks containing 28 L of the same type of medium for another week before the test. Individual 8 L glass tanks containing 4 L of the same medium were sequentially prepared to monitor animals individually after dosing. Animal care and maintenance followed the animal protocol approved by the Institutional Animal Care and Use Committee of Texas Tech University (ACUC # 05049-09).

The UPD procedure for acute toxicity testing was applied. In this method animals were dosed one at a time. The starting dose for TNT was 400 mg/kg BW, and the dose progression factor of 2 was calculated based on an estimated LD_{50} of 800 mg/kg BW determined for other species (Dilley et al. 1982; Reddy et al. 2000). The starting dose for both 2,4-DNT and 2,6-DNT was 175 mg/kg BW, and the dose progression factor was 3.2 as suggested by the UPD method. If this dosage was not lethal to the animal, the following animal in the treatment regime would be exposed to an increased dose.

Animals were fasted for 24 h before dosing, and food was reintroduced 24 h after dosing; body weights were recorded before, during, and after the test. Animals were exposed to a single dose of TNT, 2,4-DNT, and 2,6-DNT separately using oral gavage. This route of exposure fit the requirements of the OECD (2001). Animals were monitored constantly by either direct observation during the first hours or a camera system installed to record the animals' symptoms overnight. Each animal was then evaluated daily and dosed in 48-h intervals. The symptoms evaluated included: changes in respiratory rhythm, decrease in motor activities, salivation, muscle tone changes, gastrointestinal changes, skin color changes, and ocular signs. These signs were recorded during the first hours by direct observation and in comparison to the control animal. Testing continued until one of the following criteria was met: "Three consecutive animals survive at the upper bound; five reversals occur in any six consecutive animals tested; or at least four animals have followed the first reversal and specified likelihood-ratios exceed the critical values" (OECD 2001).

Moribund animals and animals at the end of the experiment, by day 14th, were euthanized using 3-aminobenzoic acid ethyl ester (MS-222) purchased from Sigma-Aldrich (St. Louis, MO, USA) at a concentration of 3 mg/L. After euthanasia animals were rinsed with deionized (DI) water, then weighed and dissected. The weight of the following tissues was recorded: kidney, liver, spleen, and heart. Gross morphological analysis of collected organs was performed using a stereomicroscope Motic K400 from Martin Microscope (Easley, SC, USA).

The LD_{50} and 95% confidence intervals were calculated using the maximum likelihood method (OECD 2001). For this study, the AOT425StatPgm program created by the Environmental Protection Agency (EPA) (2003) facilitated the calculations. The R statistic program (R, version 1.9, R development Core team) was used to calculate linear regression analysis for the relative weight of organs and the measured dose administered in the acute study. Alpha was set at 0.05 to determine the significance of the test.

Results and Discussion

Calculated dosages for TNT were 400, 800, and 2,000 mg/kg. Four of four animals died in the highest dose group. Dosages for 2,4-DNT, and 2,6-DNT were 175, 500 and 2,000 mg/kg. Three of three animals died at the highest dose group. Dose progression for TNT, 2,4-DNT, and 2,6-DNT are mentioned in Tables 1, 2, and 3, respectively. There was no lethality among PEG-treated frogs; all controls were dosed with 2,000 mg/kg BW of PEG. The LD_{50} value for TNT was

Table 1 Dose sequence, initial body weight, and outcome of male adult bullfrogs acutely exposed to TNT (single oral exposure)

Dose in mg/kg BW (number of animals dosed)	Initial BW (g)	Outcome/time to death in days/BW (g) for early deaths	Outcome/BW (g) at day 7	Outcome/BW (g) at day 14
Control (1)	165	O	O/168	O/170
400 (1)	180	O	O/172	O*/153
800 (1)	169	O	O/167	O*/148
2,000 (1)	164	O	X/166	X
800 (1)	170	O	O/168	O*/161
2,000 (1)	182	X/6/164	X	X
2,000 (1)	150	X/3/136	X	X
2,000 (1)	165	O	X/159	X

O = survived, X = died, * = animals that survived and were euthanized at day 14

Table 2 Dose sequence, initial body weight, and outcome of male adult bullfrogs acutely exposed to 2,4-DNT (single oral exposure)

Dose in mg/kg BW (number of animals dosed)	Initial BW (g)	Outcome/time to death in days/BW (g) for early deaths	Outcome/BW (g) at day 7	Outcome/BW (g) at day 14
Control (1)	131	O	O/133	O/135
175 (1)	141	O	O/133	O*/125
500 (1)	136	O	O/120	O*/110
2,000 (1)	110	X/2/125	X	X
500 (1)	135	O	O/119	O*/115
2,000 (1)	158	X/5/139	X	X
500 (1)	137	O	O/121	O*/108
2,000 (1)	103	X/2/132	X	X

O = survived, X = died, * = animals that survived and were euthanized at day 14

Table 3 Dose sequence, initial body weight and outcome of male adult bullfrogs acutely exposed to 2,6 -DNT (single oral exposure)

Dose in mg/kg BW (number of animals dosed)	Initial BW (g)	Outcome/time to death in days/BW (g) for early deaths	Outcome/BW (g) at day 7	Outcome/BW (g) at day 14
Control (1)	185	O	O/186	O/190
175 (1)	230	O	O/228	O*/218
500 (1)	206	O	O/230	O*/218
2,000 (1)	186	X/1/196	X	X
500 (1)	182	O	O/252	O*/207
2,000 (1)	178	X/3/198	X	X
500 (1)	192	O	O/218	O*/205
2,000 (1)	173	X/3/190	X	X

O = survived, X = died, * = animals that survived and were euthanized at day 14

determined to be 1,060 mg/kg BW with an approximate 95% confidence interval ranging from 800 to 2,000 mg/kg BW. The LD_{50} value for 2,4-DNT and 2,6-DNT was 1,098 mg/kg BW with an approximate 95% confidence interval ranging from 550 to 2,000 mg/kg BW.

Clinical symptoms observed in the high dose group of TNT, 2,4-DNT, and 2,6-DNT included: changes in respiratory rhythm (dyspnea, cyanosis, and tachypnea), decrease in spontaneous motor activities (somnolence, loss of righting reflex, prostration, tremors, tonic and clonic convulsion), salivation, muscle tone changes (hypertonia, hypotonia), gastrointestinal changes (vomiting, orange urine excretions), changes in skin color, skin pigmentation, and ocular signs (relaxation of the nictitating membrane). All of the symptoms previously mentioned were adopted from acute toxicity symptoms in animal studies (Hayes 1994).

Compared to the two DNT isomers, TNT showed delayed toxicity. Animals dosed at 2,000 mg/kg BW of TNT died within an average of 138 h. However, frogs exposed to 2,000 mg/kg BW of 2,6-DNT died within an average of 72 h, that is 8 h faster than frogs exposed to 2,000 mg/kg BW of 2,4-DNT. Toxicity symptoms for the animals exposed to 2,000 mg/kg BW started within the first

2 h after dosing. Symptoms before seizures included increased respiratory rate, salivation, stomach discomfort showed by stretching hind legs, adopting a standing position by resting front legs on the tank wall, fast movements in circles, and lethargy. Tremors and convulsions appeared after 37, 30, and 28 h of dosing for TNT, 2,4-DNT, and 2,6-DNT, respectively. In some cases, seizures were followed by a recovery period with subsequently involuntary contractions of the muscles in different parts of the body. An interesting mechanism of detoxification in amphibians was observed. Bullfrogs are able to extrude their stomachs, empty the content then pulling the empty stomach back in place. This behavior was observed three times in animals exposed to TNT compared to 2,4-DNT and 2,6-DNT exposed bullfrogs normally after 12 h of exposure.

Necropsy of animals exposed to TNT and DNT isomers revealed gross morphological changes including liver and kidney necrosis, and heart failure in the case of 2,6-DNT exposed animals. A loss of appetite was observed in animals in the 2,000 mg/kg BW dose group of all compounds tested. Frogs that were treated with 2,000 mg/kg BW of TNT, 2,4-DNT,and 2,6-DNT were greatly discolored. Frogs exposed to 2,4-DNT and 2,6-DNT displayed similar symptoms to that of TNT with the following exceptions: 2,4-DNT, and 2,6-DNT-exposed animals had a strong yellow urine color compared to the orange urine color secreted by frogs exposed to TNT. In addition, frogs exposed to 2,6-DNT apparently retained more fluid and looked bloated compared to animals exposed to 2,4-DNT and TNT.

Body weight of animals exposed to the lowest dose decreased for all the compounds tested at the initial dose during the course of the experiment (Tables 1, 2, and 3). However, the dose 2,000 mg/kg BW for 2,4-DNT and 2,6-DNT caused a moderate increase of body weight. Linear regression models did not show significant differences for any of the relative organ weights compared to doses for all the compounds tested. The TNT-treated (2000 mg/kg BW) frog that was found dead was deeply pigmented and its tongue was partially protruding through its mouth. The liver of frogs dosed with 175 mg/kg of 2,4-DNT, and 2,6-DNT appeared normal. However, all animals dosed with 2,000 mg/kg BW DNT isomers had enlarged livers that were grossly necrotic. Similarly, extensive necrosis was observed in all the kidneys of the 2,000 mg/kg BW 2,4-DNT, and 2,6-DNT dose group. Necrosis was more pronounced in animals exposed to 2,000 mg/kg BW. The lungs of frogs dosed with 2,000 mg/kg BW 2,4-DNT and 2,6-DNT were cyanotic. An increase in the size of the spleen in the order of 2 fold also was noticed in one animal dosed with 2,000 mg/kg BW TNT. Similarly, two other animals exposed at 2,000 mg/kg BW of 2,4-DNT and 2,6-DNT increased spleen weight in the order of 1.7 and 5.4 fold, respectively. Qualitative estimation of the bile coloration in animals exposed to a high concentration of 2,000 mg/kg BW for all the compounds tested was yellow while the animals exposed to 175 mg/kg BW and 400 mg/kg BW showed a green coloration.

DNT isomers and TNT acute toxicity symptoms were similar at the highest dose. Observed symptoms of acute and subacute toxicity included, but were not limited to, prostration, vomiting, salivation, lethargy, increased respiratory rate, convulsion and tremors, orange urine in the case of TNT, loss of righting reflex, loss of appetite, and death. Seizures and mild convulsions after 1–2 h, orange urine and lethargy, ataxia, and increased respiratory rate have also been reported in other species, namely rats and mice acutely exposed to TNT (Dilley et al. 1982; Reddy et al. 2000). Ataxia and slight cyanosis was observed in rats and mice exposed to 2,4-DNT (Lane et al. 1985). Some of the signs of acute toxicity for 2,4-DNT in Northern bobwhite were similar to the ones observed in bullfrogs such as weight loss and lethargy (Johnson 2005).

The calculated LD_{50} for TNT in bullfrogs is higher than the LD_{50} determined for mice (600 mg/kg BW) (Dilley et al. 1982) and lower than the LD_{50} in male rats, which was calculated to be 1,320 mg/kg BW (Dilley et al. 1982). The Northern bobwhite appears to be more sensitive to 2,4-DNT with an LD_{50} of 55 mg/kg BW (Johnson 2005), which is almost 20 times less than the LD_{50} for bullfrogs. The LD_{50} reported in mice after administration of 2,4-DNT ranged from 1,340 to 1,954 mg/kg BW (Ellis et al. 1978; Lee et al. 1975; Vernot et al. 1977), which is near the upper limit confidence interval of 2000 mg/kg BW calculated for the LD_{50} in bullfrogs.

Once TNT and DNT enter the environment and because of their relatively high solubility and low octanol water partitioning coefficient there is potential for transport in aquatic ecosystems. In surface waters biological and photolytic degradation occurs leaving low concentrations of these compounds available for wildlife. TNT has been detected at concentrations of 774–998 μg/L in lagoon water (Triegel et al. 1983) and 1–178 mg/L from load, assemble, and packing plants (LAP) (Patterson et al. 1977). Both 2,4-DNT and 2,6-DNT were found as major components in wastewaters from TNT manufacturing facilities (Spanggord and Suta 1982; Spanggord et al. 1982) at concentrations ranging from 0.04 to 48.6 mg/L and 0.06 to 14.9 mg/L for 2,4 and 2,6-DNT, respectively. Thus, LD_{50} results, relative low concentrations in aquatic environments, and short half-lives of these compounds indicate that adult bullfrogs in the wild are at low risk for either exposure or toxicity.

Both TNT and DNT isomers caused nominal reduction in the body weight of the frogs dosed at the initial concentration over the duration of the study. The largest

reduction in weight was seen in frogs dosed with 400 mg/kg BW of TNT in the order of 15%. In contrast, frogs dosed at 500 mg/kg BW with 2,6-DNT looked bloated and increased weight by approximately 9%. Animals dosed at 2,000 mg/kg BW with 2,4-DNT; 2,6-DNT, and TNT showed slight weight gain, which can be an effect of accumulation of great amount of liquid found at necropsy that was observed in all the animals that gained BW. In animals exposed to 2,6-DNT, coagulated blood was also found in the body cavity. Linear regression analysis of relative organ weights showed no significant change for the acute study. Relative tissue weights of spleen and liver for two frogs dosed at 2,000 mg/kg BW were higher than any other frog tested; however, p value greater than 0.05 indicated no significant difference.

TNT and 2,4-DNT, and 2,6-DNT have similar acute toxicity in male bullfrogs. The median lethal dose for TNT was 1060 mg/kg BW while the LD_{50} for both 2,4-DNT and 2,6-DNT was 1098 mg/kg BW. All compounds tested caused alterations of the Central Nervous System (CNS). Changes in the respiratory and circulatory systems were also detected. Liver and kidney necrosis were the most common gross internal morphology changes, as well as an increase in spleen and liver size. External gross morphology changes included changes in urine color, skin color and body weight.

Acknowledgements The authors greatly appreciate the collaboration of Mike Wages and Colton Wilson for their assistance during the dosing period. We also thank Dr. Wanda Goleman and Dr. Jaclyn Cañas for their helpful comments. This research was financially supported by the Strategic Environmental and Research Development Program (SERDP) project T05-07.

References

Dilley JV, Tyson CA, Spanggord RJ, Sasmore DP, Newel GGW, Dacre JC (1982) Short term oral toxicity of 2,4,6 TNT and RDX mixture in mice, rats, and dogs. J Toxicol Environ Health 9:587–610

Ellis HV, Hodgson JR, Hwang SW (1978) Mammalian toxicity of munitions compounds. Phase I. Acute oral toxicity, primary skin and eye irritation, dermal sensitization, disposition and metabolism and Ames tests of additional compounds. Progress report No. 6. Midwest Research Institute, Kansas City, MO. Contract no. DAMD 17-74-C-4073, AD A069 444

Hayes AW (1994) Principles and methods of toxicology. Raven, New York

Lane RW, Simon GS, Dougherty RW (1985) Reproductive toxicity and lack of dominant lethal effects of 2,4-dinitrotoluene in the male rat. Drug Chem Toxicol 8:265–280. doi:10.3109/01480548509038650

Lee CC, Dilley JV, Hodgson JR (1975) Mammalian toxicity of munition compounds: Phase I. Acute oral toxicity, primary skin and eye irritation, dermal sensitization, and disposition and metabolism. Report No. 1, Project No. 3900-B. Midwest Research Institute, Kansas City, MO, Contract DAMD17–74-c-4073

Johnson MS, Michie MW, Bazar MA, Gogal RM (2005) Influence of oral 2,4-DNT exposure to the northern bobwhite (*Colinus virginianus*). Int J Toxicol 24:265–274. doi:10.1080/10915810591007247

Organization for Economic Cooperation and Development (OECD) (2001) Guideline for testing of chemicals. http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/OECD/OECD_GL420.pdf

Environmental Protection Agency (EPA) (2003) User documentation for the AOT425StatPgm program prepared for the USEPA by Westat. http://www.epa.gov/oppfead1/harmonization/docs/aot425manual.pdf

Reddy G, Chandra SAM, Qualls CW Jr (2000) Toxicology of 2,4,6-TNT in hispid cotton rats (*Sigmodon hispidus*): hematological, biochemical, and pathological effects. Int J Toxicol 19:1–9

Rispin A, Farrar D, Margosches E, Gupta K, Sitzel K, Greene M, Meyer W, MCall D (2002) Alternative methods for median lethal dose (LD_{50}) test: the up-and-down procedure for acute oral toxicity. ILAR J 43:233–243

Patterson JW, Shapira NI, Brown J (1977) Pollution abatement in the military explosives industry. Proc 31st Ind Waste Conf 31:385–394

Smith NJ (2007) Age dependent acute oral toxicity of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and two anaerobic N-nitroso metabolites in Deer mice (*Peromyscus maniculatus*). Chemosphere 67:2267–2273

Spanggord RJ, Gibson BV, Keck RG (1982) Effluent analysis of wastewater generated in the manufacture of 2,4,6-trinitrotoluene. Environ Sci Technol 16:229–232

Spanggord RJ, Suta BE (1982) Effluent analysis of wastewater generated in the manufacture of 2,4,6-trinitrotoluene. Determination of a representative discharge of ether extractable components. Environ Sci Technol 16:233–236

Triegel EK, Kolmer JR, Ounanian DW (1983) Solidification and thermal degradation of TNT waste sludge using asphalt encapsulation. In: National conference on management of uncontrolled hazard waste sites, October 31–November 2 1983, Silver Spring, MD: Hazardous Materials Controls Research Institute, Washington, pp 270–274

Vernot EH, MacEwen JD, Haun CC (1977) Acute toxicity and skin corrosion data for some organic and inorganic compounds and aqueous solutions. Toxicol Appl Pharmacol 42:417–423