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Acute toxicity of injected drugs and substances in fish

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ABSTRACT

This protocol, modified from OECD 203 (Acute toxicity test, adult fish), tests toxic (lethal and nonlethal) effects of substances that have been injected intraperitoneally in adult fish. While OECD 203 is appropriate for testing the effects of waterborne substances (e.g., sewage effluents, pesticides, and other toxicants that can reach water bodies), the modified version can be added in a drug screening pipeline

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KEYWORDS

Acute toxicity, Fish toxicity tests, Drug screening, Mortality

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GUIDELINES

This protocol is based on OECD 203, "Fish, Acute toxicity testing". As a result, guidelines for reducing the number of animals that are used in testing, as well as guidelines regarding substance safety (e.g., use of personal protective equipments [PPE], reference to MSDS of reagents and solvents, etc.) should be followed.

MATERIALS

NAME	CATALOG #	VENDOR
Aquarium thermometer		
Eutech™ DO 450 Dissolved Oxygen Meter	ECDOWP45000	Thermo Fisher
pH meter		

NAME	CATALOG #	VENDOR
Tanks made of chemically inert material		

MATERIALS TEXT

While the test chemical will be injected and not dissolved in the water, as in OECD 203, the use of tanks made of chemically inert material is important because animals can eliminate the substance in the water through urine or gill excretion. Any glass, polypropylene, or stainless steel tank can be used; however, silicone is known to have a strong capacity of absorbing lipophilic chemicals, and therefore glass tanks using silicone seals should be avoided.

SAFETY WARNINGS

Guidelines regarding substance safety (e.g., use of individual protective equipments, reference to MSDS of reagents and solvents, etc.) should be followed. While substances which are going to be tested probably have no known toxic effects or mechanisms, care should be taken to reduce contact by using safety googles, gloves, and PPE throughout the protocol.

BEFORE STARTING

Principle of the test

Test fish are injected with the test chemical, and lethal and sub-lethal endpoints (visible abnormalities related to appearance and behavior) are recorded at specific time intervals after injection (5-6 h, 24 h, 39 h, 48 h 54 h, 72 h, 78 h, and 96 h after injection). Where possible, lethal doses capable of killing 50% of the fish are recorded (LD_{50}).

Test validity

For the test to be valid, the following conditions should hold:

- In the control group (vehicle-injected animals), the mortality should not exceed 1 fish 96 h after injection;
- Water parameters should not fall below critical values for the chosen species.

Introducing the test in a pipeline

Dose-response curves for a single behavioral test can be derived by injecting the fish with the test chemical and evaluating its effect 30 min after testing. Animals are then transferred to holding tanks, and toxicity is observed for the time intervals proposed above.

Choosing doses

When selecting a range of test doses, all sources of information should be considered, including whether the chemical has been tested in other species. In the absence of such information, a rule-of-thumb is to use the dose range from OECD 423 (Acute Oral Toxicity), i.e., 5, 50, 300, and 2000 mg/kg.

Sample sizes and stepwise procedure

Since the protocol proposes a stepwise approach to testing, final sample sizes will vary. Nonetheless, minimum sample size will always be 6 animals (3 in the control group, 3 in the lowest dose, considering that the lowest dose already produces lethality), and the maximum sample size will be 26 animals. What determines the final sample size is the results from the stepwise procedure, described in the protocol.

Alternative routes of administration

If the intent is to accelerate drug discovery, sometimes using oral administration is preferable. Doing so in small fish (especially zebrafish, currently the species of choice in the field) is difficult, but a protocol is available by Collymore et al. (2013; https://www.jove.com/t/50691/gavaging-adult-zebrafish).

Testing conditions

- **Total duration:** 96 hs
- **Lighting:** Should fall within photoperiod ranges of the test species (e.g. , for zebrafish, 14L:10D). Light intensity should be between 540-1000 lux (10-20 µE/m^2/s, or 50-100 ft-c)
- **Water temperature:** Should not vary more than 2 ºC between test tanks or between successive days at any time during testing, and should be within the temperature range specified for the test species. Remind that toxic effects, especially mortality, can be synergistically increased by higher temperatures. For zebrafish, a range of 21 ºC - 25 ºC is preferable.

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- **Oxygen concentration:** No smaller than 60% of the air saturation value.
- **Feeding:** Following protocols for intraperitoneal injection (Kinkel et al., 2010; https://www.jove.com/t/2126/intraperitoneal-injection-into-adult-zebrafish), fish should be fasted for at least 24 h before injections.
- **Room disturbances:** Disturbances from excessive vibration or noise should be avoided, as these can lead to changes in behavior.

Anesthesia and injection procedures

1 Anesthesize fish in ice-cold water (12-14 ºC). Remove animal from water as soon as movements stop. **On ice**

2 Transfer animal to surgical bed (water-soaked sponge with a cut to fixate the fish), kept in a vessel with ice-cold water. **On ice**

3 ⚠

Inject the chemical, using the protocol by Kinkel et al. (2010).

> Mary D. Kinkel, Stefani C. Eames, Louis H. Philipson, Victoria E. Prince (2010). Intraperitoneal Injection into Adult Zebrafish. Journal of Visualized Experiments. http://10.3791/2126

> **Recommended injection volumes are of 1 µL/0.1g body weight.** Using a 10 µL microsyringe is useful in the case of small fish, such as zebrafish.

4 Gently remove animal from the surgical bed and return to observation tank, or leave to rest until behavioral experiment.

4.1 Repeat injection procedures in 2 animals, for a total of 3 animals per dose.

Behavioral experiment

5 ✳

Optionally, running a behavioral experiment 30 min after injection can decrease the number of animals used in a drug screening pipeline. We commonly screen chemicals for anxiolytic-like effects using the light/dark preference test and/or the novel tank test.

Observe lethal and sublethal endpoints 4d

6 Register lethal and sublethal endpoints at the following time points after injection:

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- 2.5 h
- 5.5 h
- 24 h
- 30 h
- 48 h
- 54 h
- 72 h
- 78 h
- 96 h

7

Mortality: Definition of "mortality" is derived from OECD 203: "Fish are considered dead if there is no visible movement (e.g. gill movements) and if touching of the caudal peduncle produces no reaction. Mortalities are recorded, and dead fish are removed as soon as they are observed".

OECD. Test No. 203: Fish, Acute Toxicity Test. OECD Guidelines for Testing of Chemicals. Section 2: Effects on biotic systems.
http://10.1787/9789264069961-en

8 Register sublethal endpoints, as follows:

Domain	Clinical sign
Distribution	Loss or densing of schooling / shoaling behavior
Vertical distribution - Surfacing or bottom-dwelling	
Equilibrium and buoyancy	Abnormal horizontal orientation
Abnormal vertical orientation	
Loss of buoyancy control	
Observed behaviors	Hypoactivity or hyperactivity
Spiral swimming	
Hyperventilation or hypoventilation	
Irregular ventilation	
Increased ventilation depth	
Convulsions	
Coughing, gulping, or gasping	
Surface escape / avoidance behaviors	
Bottom escape / avoidance behaviors	
Irritated skin behaviors	
Aggression and/or cannibalism	
Appearance	Tetany
Skin color - Darkening, lightening, or mottled skin	
Oedema	
Hemorrhagic areas or petechiae	

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Exophthalmia	
Mucus secretion	
Faecal casts	
Provoked behavior	Visual and tank knocking stimulus - over reactivity or under reactivity
Tactile stimulus - over reactivity or under reactivity	

Bruna Patricia Dutra Costa, Layana Aquino Moura, Sabrina Alana Gomes Pinto, Monica Lima-Maximino, Caio Maximino. Zebrafish Models in Neural and Behavioral Toxicology across the Life Stages. Fishes.
http://10.3390/fishes5030023

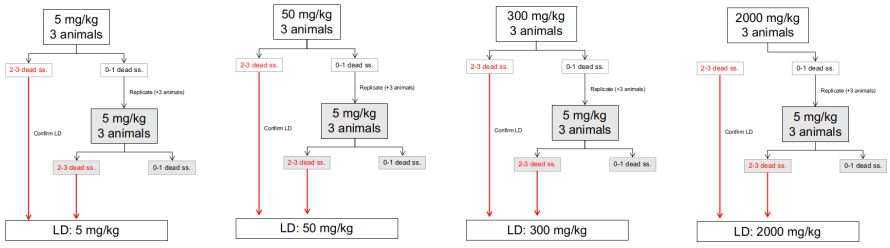
9 Humanely sacrifice animals after the last observation (i.e., 96 h after injection).

9.1 ✱

After sacrifice, animals can be dissected, and organs be harvested for pathology.

Stepwise procedure

10 After running the control (vehicle) group, proceed to inject animals with the lowest dose and follow the stepwise procedure:



10.1 Inject 3 animals with the lowest dose (e.g., 5 mg/kg) and repeat steps 7-9, above. **go to step #7**

10.2 If, at the end of the last time interval (96 h), 0-1 animals are dead, replicate the experiment with 3 more animals to confirm non-lethality. If 2-3 animals are dead, proceed to determine the dose as a lethal dose.

10.3 If non-lethality is confirmed, inject 3 animals with the next dose (e.g., 50 mg/kg), and repeat steps 7-9. **go to step #7** . At the end of the last time interval for this dose, determine mortality (**go to step #10.2**)

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10.4 ⚠

Stop experiment when the lethal dose is reached. No more animals must be exposed to the test chemical once the dose is established.

Results should be reported as:

- Mortality in the control(s)
- The LD value at 96 h
- Incidence and description of visible abnormalities, as describd in step 8

go to step #8

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