Neurotoxicology 74 (2019) 67–73

Contents lists available at ScienceDirect

Neurotoxicology

journal homepage: www.elsevier.com/locate/neuro

Full Length Article

Determination of narcotic potency using a neurobehavioral assay with larval zebrafish

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ARTICLE INFO

Keywords:
Zebrafish
Narcosis
Neurobehavioral effects
Locomotor activity
Toxicity
Etomidate
Ethanol
Alcohols
Surfactants

ABSTRACT

Background: Identifying chemicals with narcotic potency is an important aspect of assessing the safety of consumer products that may be accidentally ingested. A rapid and efficient assay of narcotic potency is desired for assessing chemicals with such suspected activity.

Objectives: This purpose of this research was to develop a non-mammalian vertebrate, high throughput, neurobehavioral method to assess the narcotic potency of chemicals using larval zebrafish.

Methods: Larval zebrafish were acutely exposed to chemicals beginning at 5 days post fertilization (5 dpf). Locomotor activity, elicited by regular, periodic photostimulation, was quantified using a video tracking apparatus. Narcotic potency was determined as the molar concentration at which photostimulated locomotor activity was reduced by 50% (IC_{50}). Toxicity was assessed based on observations of morbidity or mortality. Recovery was assessed following removal of test material by serial dilution and reassessment of photostimulated behavior 24 hr later (6 dpf).

Results: A total of 21 chemicals were assessed. Etomidate, a human narcotic analgesic agent, was used as a reference material. Investigating a series of eleven linear, primary alcohols (C6 to C16), a relationship between narcotic potency and carbon number was observed; narcotic potency increased with carbon number up to C12, consistent with historical studies. For a set of technical grade surfactants, nonionic surfactants (i.e., alcohol ethoxylates) were observed to be narcotic agents while anionic surfactants produced evidence of reduced locomotor activity only in combination with toxicity. Of the solvents evaluated, only ethanol exhibited narcotic activity with an IC_{50} of 261 mM and was the least potent of the chemicals investigated. Etomidate was the most potent material evaluated with an IC_{50} of 0.39 µM.

Conclusions: The larval zebrafish neurobehavioral assay provides a method capable of estimating the narcotic potency of chemicals and can identify if toxicity contributes to observed neurobehavioral effects in the test organism.

1. Introduction

Accidental ingestions are a concern for many types of consumer products. In 2015, 2.1 million human exposures to both pharmaceutical and non-pharmaceutical products were reported to poison control centers in the U.S. (Mowry et al., 2016). Of these, 78% (or 1.7 million) were unintentional or accidental. Of these unintentional/accidental exposures, 62% (or 1.0 million) involved children less than 6 years of age. For children less than 6 years of age, 11.2% (or 118,346) of reported accidental exposures involved household cleaning products. The large majority of these exposures were via the oral route.

Household cleaning products may contain chemicals with the potential to produce anesthetic/narcotic like effects. The oral administration of solvents and some classes of non-ionic surfactants (i.e., alcohol ethoxylates) to experimental animals have been shown to elicit anesthetic/narcotic like effects including loss of righting reflex, respiratory depression, muscle paralysis, and CNS depression (McCreery and Hunt, 1978; Rang, 1960; Talmage, 1991; Zerkle et al., 1987). These effects are analogous to observations of drowsiness, lethargy, or non-responsiveness reported in emergency treatment situations following exposures to products containing these ingredients. Thus, it is important to identify ingredients with narcotic potential in order to assess

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https://doi.org/10.1016/j.neuro.2019.05.005
Received 7 November 2018; Received in revised form 4 April 2019; Accepted 7 May 2019
Available online 11 May 2019
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Table 1
The 21 chemicals utilized in this study with the chemical name, synonym, CAS number, chemical source, and selected physical properties.

Chemical Name	Synonym	Class	CAS RN	Source	Purity	MW, g/mol	Water Solubility
Ethanol	C2-OH	Alcohol/Solvent	64-17-5	Pharmaco-Aaper	≥ 99.9%	46.07	Fully miscible
1-Hexanol	C6-OH	Alcohol	111-27-3	Sigma-Aldrich	≥ 97.5%	102.17	5900 mg/L at 20 °C
1-Heptanol	C7-OH	Alcohol	111-70-6	Sigma-Aldrich	≥ 97.5%	116.20	1313 mg/L at 20 °C
1-Octanol	C8-OH	Alcohol	111-87-5	Sigma-Aldrich	≥ 99.0%	130.23	551 mg/L at 25 °C
1-Nonanol	C9-OH	Alcohol	143-08-8	Sigma-Aldrich	≥ 97.5%	144.25	128 mg/L at 20 °C
1-Decanol	C10-OH	Alcohol	112-30-1	Sigma-Aldrich	≥ 98.0%	158.28	39.5 mg/L at 25 °C
1-Undecanol	C11-OH	Alcohol	112-42-5	Sigma-Aldrich	≥ 98.5%	172.31	8 mg/L at 20 °C
1-Dodecanol	C12-OH	Alcohol	112-53-8	Sigma-Aldrich	≥ 97.5%	186.33	1.93 mg/L at 20 °C
1-Tridecanol	C13-OH	Alcohol	112-70-9	Sigma-Aldrich	≥ 96.5%	200.36	0.38 mg/L at 20 °C
1-Tetradecanol	C14-OH	Alcohol	112-72-1	Sigma-Aldrich	≥ 96.5%	214.39	0.191 mg/L at 25 °C
1-Pentadecanol	C15-OH	Alcohol	629-76-5	Sigma-Aldrich	≥ 98.5%	228.41	0.102 mg/L at 25 °C
1-Hexadecanol	C16-OH	Alcohol	36653-82-4	Sigma-Aldrich	≥ 98.5%	242.44	0.013 mg/L at 25 °C
Glycerol	Glycerol	Solvent	56-81-5	P&G	≥ 99.0%	92.09	Miscible
1,2-Propanediol	1,2-Propanediol	Solvent	57-55-6	Dow	≥ 99.0%	76.09	Miscible
Poly(oxy-1,2-ethanediyl), α-dodecyl-ω-hydroxy-	C12-EO9	Alcohol Ethoxylate	9002-92-0	Sigma-Aldrich	≥ 99.0%	583	Soluble (> 10 g/L)
Alcohols, C12-16, ethoxylated	C12-16-EO7	Alcohol Ethoxylate	68551-12-2	P&G	≥ 97.0%	517	Soluble (> 10 g/L)
Alcohols, C12-16, ethoxylated	C12-14-EO9	Alcohol Ethoxylate	68551-12-2	P&G	≥ 99.0%	605	Soluble (> 10 g/L)
Alcohols, C10-16, ethoxylated	C14-15-EO7	Alcohol Ethoxylate	68002-97-1	P&G	≥ 99.0%	542	Slightly Soluble (> 1 g/L)
Benzenesulfonic acid, 4-C10-13-sec-alkyl derivs.	C10-13-LAS	Linear Alkylbenzene Sulfonate	85536-14-7	P&G	≥ 98.0%	322	Soluble (> 10 g/L)
Poly(oxy-1,2-ethanediyl), α-sulfo-ω-(dodecyloxy)-, sodium salt (1:1)	C11-15-AE3S	Alcohol Ethoxy Sulfate	9004-82-4	P&G	70.0%	427	Soluble (> 10 g/L)
Etomidate	Etomidate	Anesthetic	33125-97-2	Sigma-Aldrich	≥ 98.0%	244.29	63.2 mg/L

the likelihood that a formulation may produce narcotic-like symptoms following accidental ingestion.

The term narcosis itself is imprecisely defined with a number of meanings. For clarity and purposes herein, narcosis is defined as a "general and nonspecific reversible depression of neuronal excitability, produced by a chemical or physical agent, usually resulting in stupor rather than in anesthesia (Steadman's Medical Dictionary)". Thus, a narcotic agent is a chemical or physical agent capable of producing a state of narcosis.

The study of sedation, narcosis, and anesthesia is traditionally completed in mammalian (e.g., rodent and nonhuman primate) model systems given the physiological complexity of this event (Brown et al., 2011; Chau, 2010; Franks, 2008; Hemmings et al., 2005; Humphrey et al., 2002). Aquatic models are also used to study the narcotic action of chemicals, especially as it relates to mechanisms of aquatic toxicity (Veith and Broderius, 1990; Veith et al., 2015). Although these existing models are useful, they are expensive and often time consuming to implement. Moreover, there has been a shift in toxicological hazard identification to implement the three R's: 1) replace animal use with alternative techniques, 2) reduce the number of animals and 3) refine the way the experiments are carried out (Fenwick et al., 2009; Hartung, 2009). With this paradigm shift, there is a need for an alternative testing model that is able to rapidly assess and evaluate the potential of chemicals to produce complex, system-dependent effects such as narcosis.

The early life-stage zebrafish was selected as a model organism to develop a high throughput neurobehavioral assay for the study of narcosis potential. Zebrafish develop rapidly, have a genome that is 80% similar to humans and accurately model a number of human disease states (Howe et al., 2013). The signal transduction mechanisms, anatomy, and physiology of zebrafish are homologous to those of humans (Barbazuk et al., 2000; Nguyen et al., 2013; Rinkwitz et al., 2011). Zebrafish possess all the classical sense modalities found in humans and have been used to study pharmacological and toxicological responsiveness in models of human disease (Goldsmith, 2004; Hill et al., 2005; Moorman, 2001). Additionally, central nervous system development and neurogenesis are highly conserved between zebrafish and humans (Shams et al., 2017). Finally, the locomotor response in zebrafish can be easily stimulated by changing lighting conditions (Burgess and Granato, 2007; Guo et al., 2015; Irons et al., 2010; Padilla et al., 2011; Saili et al., 2012), which provides a whole organism test system that can rapidly evaluate multiple concentrations of a test material using a 96-well platform.

Locomotor activity was selected as the endpoint for these studies. Motor activity in general is an endpoint with an extensive history of use in pharmacology studies (Lynch et al., 2011; Porsolt et al., 2002; Roux et al., 2005). For example, the righting reflex in rodents is an endpoint used in the study of anesthetic agents and correlates well with loss of consciousness in humans (Franks, 2008). Locomotor activity has been used to study the effects of anesthetic agents in numerous species including worms, flies, and zebrafish (Humphrey et al., 2007; Morgan et al., 2014; Renier et al., 2007; Zalucki and van Swinderen, 2016). Thus, locomotor activity is a preferred endpoint in that it allows results to be compared across multiple species including humans.

In the present study, an experimental protocol was developed using larval zebrafish for the assessment of narcosis potential. The protocol is designed following principles of inhibition studies where the effect of an inhibitor (i.e., a narcotic agent) is assessed against a stimulated activity (i.e., locomotor activity). Locomotor activity is stimulated using a series of light-to-dark phototransition periods and quantified using a video tracking apparatus. At 5 days post-fertilization (dpf), larvae are exposed to a concentration series of a potential narcotic agent and a concentration-response curve is generated against stimulated locomotor activity. The outcome is a quantitative measure of narcotic activity, i.e., the inhibitory concentration at which stimulated locomotor activity is reduced by half (IC_{50}). This is followed by immediate removal of the test chemical by serial dilution and a phenotypic screen to assess the larvae for toxicity outcomes. At 6 dpf, the neurobehavioral assay and phenotypical screen are repeated to confirm that the narcosis effects observed at 5 dpf were reversible and that treated larvae revert to locomotor and phenotypical responses typical of untreated larvae in control incubation media.

Using this assay in a high throughput format, a total of 21 chemicals were assessed, including a series of alcohols, surfactants, and a known human narcotic analgesic agent. The chemicals were selected based on previous reports of narcosis potential and/or known usage in consumer cleaning products. Through optimization, a high throughput neurobehavioral assay in larval zebrafish is presented that has the capability to provide important information on the narcosis potential of chemicals.

2. Materials and methods

2.1. Chemicals

The chemicals were provided neat and dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions for serial dilution. Table 1 lists the chemicals used in this study, their synonyms, CAS registry number, and supplier.

2.2. Zebrafish care

Tropical 5D (Plant City, FL) zebrafish (*Danio rerio*) were reared at the Sinnhuber Aquatic Research Laboratory, Oregon State University. Adult fish were maintained in a recirculating water system with a water temperature of 28 ± 1 °C and a 14 h light:10 h dark lighting schedule. Adult fish were fed GEMMA Micro 300 or 500 (Skretting, Inc., Fontaine Les Vervins, France) twice a day (Barton et al., 2016). All embryos used in exposure experiments were collected following group spawning of adult zebrafish as described previously (Barton et al., 2016; Kimmel et al., 1995; Westerfield, 2007). Briefly, zebrafish were housed in densities of ˜500 fish/50-gallon tank at 28 °C in recirculating water supplemented with Instant Ocean salts (Spectrum Brands, Blacksburg, VA) to create a salinity of 600 microsiemens. Sodium bicarbonate was added as needed to adjust the pH to 7.4. The embryos were collected using a spawning funnel, staged, and maintained in an incubator at 28 °C. Fish husbandry, reproductive techniques, and larval zebrafish assays were all conducted according to Institutional Animal Care and Use Committee protocols at the Sinnhuber Aquatic Research Laboratory, Oregon State University.

2.3. Exposure protocol

To distinguish between potential narcotic-like effects (narcosis) and any general toxicity effects, the assay is conducted over two days (Fig. 1). Zebrafish embryos were enzymatically dechorionated at 4 hours post fertilization (hpf), which made them amendable to utilization of robotic placement of single embryos into individual wells of a 96-well round-bottom plate (Mandrell et al., 2012). Each well was prefilled with 100 μL of embryo medium (EM) using a 96-well Rainin Liquidator (Mettler-Toledo Rainin, Oakland, CA). Embryo media consisted of 15 mM NaCl, 0.5 mM KCl, 1 mM MgSO4, 0.15 mM KH2PO4, 0.05 mM Na2HPO4 and 0.7 mM NaHCO3 (Westerfield, 2007). After automated embryo placement systems deposited embryos into the wells, the plates were sealed with a biocompatible silicone PCR film to prevent evaporation. The plates were then placed in a stand-alone incubator at 28 ± 1 °C until 5 dpf. At 5 dpf, any dead or abnormally developed embryos are replaced with normal embryos prior to chemical exposure. Note that the larvae used to replace any dead/malformed larvae at 5 dpf were held under same conditions as rest of the larvae in the experiment (i.e., in a 96-well plate) to minimize any effects on behavior.

For each chemical, a wide range of concentrations is evaluated (12-point concentration response, n = 8 larvae per concentration, dilution factor of 1.26 for concentration spacing). Chemical stocks are made at 1000X stock solutions in DMSO and diluted in embryo media to make a 2X working solution for each test concentration. 100 μL of the 2X solutions are added to all 96 wells simultaneously using the 96-well Rainin Liquidator to ensure consistent timing between wells, bringing the final volume in each well to 200 μL. The final concentration of DMSO in each well is 0.10%.

Immediately after test chemical addition, exposed plates are placed into a ViewPoint video tracking instrument (ViewPoint, Montréal, Canada) to assess for potential narcosis effects against photostimulated locomotor activity. Data acquisition begins 5 minutes after chemical exposure. The neurobehavioral portion of the assay consists of a 48-minute photomotor response test where the lights are cycled on and off every 3 minutes for a total of 16 cycles. Locomotor activity data is acquired in 6 second bins for the full assay duration of 48 minutes. At the end of the neurobehavioral assay, the chemicals are immediately washed out by serial addition and removal (5 times) of fresh EM using a 96-well liquidator (all wells manipulated simultaneously to remove any well-exposure bias), and the larval zebrafish are assessed for any phenotypic abnormalities.

Three phenotypic endpoints are evaluated: mortality, skin integrity, and heart rate. Mortality is defined as larvae without a heartbeat, skin integrity is an assessment of the presence of any abnormal fin tissue or tissue loss, and heart rate assessment is a visual check for obvious rapid or slowed heart rate. Each endpoint is scored in a binary fashion (absence/presence) and recorded in a laboratory information system, Zebrafish Acquisition and Analysis Program (ZAAP).

Afterwards, the plates are re-sealed and returned to the incubator for 24 hours at 28 ± 1 °C. At 6 dpf, the plates are returned to ZebraBox video tracking instrument and the neurobehavioral assessment is repeated. Plates are also re-screened phenotypically as described above. The two separate photomotor evaluations, one with chemical and one without, provides a means of determining whether the changes in behavior (i.e., reduced locomotor activity) are reversible, which would indicate a pharmacological effect (i.e., narcosis), or irreversible following a one-day recovery period which would indicate a toxic effect. The phenotypic screen provides additional information on the health of the larval zebrafish following acute chemical exposure and allows for an assessment of overt toxicity.

If, after completion of the initial experiment, data analysis revealed that the concentration range selected for the determination of narcosis potential did not allow for an IC_{50} value to be determined for a test material then a second experiment was conducted to extend the concentration range for that test material. Concentrations for the second experiment were selected based on results from the first experiment to both extend the concentration range but also to include test material concentrations that were tested in the first experiment. If locomotor responses were consistent across the test concentrations used in both experiments as determined by ANOVA methods, then the data were

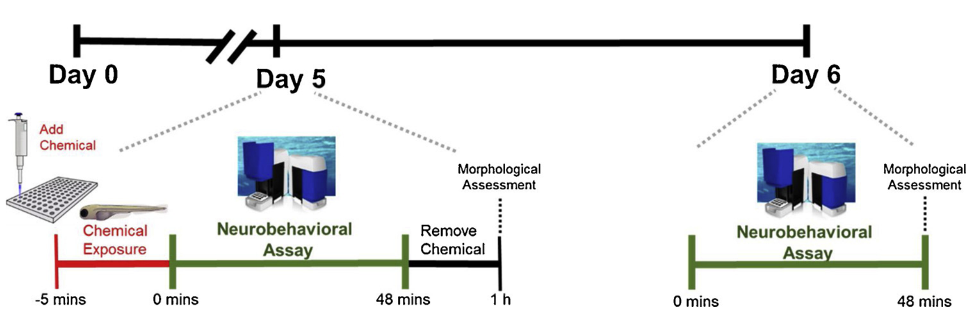

Fig. 1. Overview of experimental deign. Embryos were enzymatically dechorionated at 6 hours post fertilization (hpf) and placed one per well in a 96-well plate with 100 μL of embryo media. At 5 days post fertilization (dpf), chemicals were added to the wells with larvae and within 5 minutes placed into a neurobehavioral assay for 48 minutes. The neurobehavioral assay consists of alternating cycles of 3-minute light on and off, for a total of 16 cycles in a video tracking system. Immediately following the assay, the chemical was removed and a morphological assessment was conducted. The plates were sealed and at 6 dpf, underwent the neurobehavioral assay and morphological assessment to help distinguish absence of movement from toxicity.

combined into a single dataset for IC_{50} determination. This was done to allow for the most efficient use of zebrafish larvae and to achieve an accurate estimate of IC_{50} value.

2.4. Data analysis

IC_{50} values were estimated on locomotor activity for each dark photoperiod, i.e., photostimulated behavior, using a three-parameter logistic nonlinear regression model. If an IC_{50} by photoperiod effect was observed, an exponential decay analysis of the IC_{50} estimates was then conducted to determine the final IC_{50} for the experiment, as well as the half-time (t½) of the locomotor activity decay curve where statistical bootstrapping was used to quantify uncertainty in the final parameter estimates. Binary-response logistic regression was used to fit survival data and count data from the phenotypic assessment and calculate a toxicity value (concentration at which 50% of the test population exhibited an adverse effect or TC_{50}) and 95% confidence intervals. Locomotor activity reassessed on 6 dpf was analyzed by ANOVA methods with total locomotor activity for each test material concentration compared against control locomotor activity using Dunnett's method. Regression analysis to determine IC_{50}, $t_{1/2}$, and TC_{50} estimates was conducted using R software, whereas JMP software was used for the ANOVA data analysis (R Core Team, 2016; SAS, 2017).

3. Results

3.1. Alcohols with previously identified narcotic properties

The narcotic effects of a series of straight chain alcohols are depicted in Table 2 and Fig. 2. The narcotic effects of this series of alcohols are conserved across multiple species (i.e., mammals, fish, and invertebrates) and exhibit a well-defined structure-activity relationship based on carbon number and lipid solubility (Alifimoff et al., 1989; Anton et al., 1992; McCreery and Hunt, 1978; Rang, 1960; Roberts and Costello, 2003; Seeman, 1972). Thus, these alcohols serve as "proof-of-principle" materials to establish that the larval zebrafish neurobehavioral assay can effectively characterize the narcotic potency of chemicals. Consistent with results in other mammalian, fish, and invertebrate systems, this series of alcohols produced narcotic effects based on carbon number, exhibiting increases in narcotic potency with increasing carbon number up to C12-OH. Alcohols with carbon numbers greater than C12-OH could not be tested due to the limited water solubility of these materials. Even for the C12-OH material, it was observed that the concentration response curve flattened at concentrations greater than 20 μM and locomotor activity was not completely suppressed at higher concentrations. This contrasts with the lower carbon number alcohols where locomotor activity was completely suppressed at the upper ends of the concentration-response curves. Thus, the C12-OH material likely represents the upper end of water solubility for test materials that can be evaluated for narcotic potential in the larval zebrafish neurobehavioral assay.

An analysis of the time course data for the linear, primary alcohols indicated that these test materials have a rapid onset time for narcosis in 5 dpf larval zebrafish. The IC_{50} by time response curves were flat for alcohols C9-OH and below. No locomotor activity decay $t_{1/2}$ values could be calculated for these alcohols. It is likely that the five-minute delay between administration of chemical and start of data acquisition resulted in the time course effects of these alcohols being missed. However, beginning with the C10-OH test material, a decrease in IC_{50} values over time was noted for the first and second phototransition periods. For the C10-OH, C11-OH, and C12-OH test materials, estimated $t_{1/2}$ values (95% confidence intervals) were 140 (17–564) sec, 124 (18–2458) sec, and 83 (57–255) sec, respectively. These estimates for $t_{1/2}$ are variable due to the rapid onset of narcosis for these alcohols.

Analysis of locomotor activity at 6 dpf demonstrated that activity returned to control levels following test material removal for all of the alcohols tested. Furthermore, no adverse effects were noted for any test materials in the alcohol series up to the highest concentrations tested when assessed using the phenotypic screening criteria for toxicity.

3.2. Solvents

Three solvent test materials were assessed in the larval zebrafish neurobehavioral assay, ethanol, glycerol, and 1,2-propanediol. Neither glycerol nor 1,2-propanediol produced responses indicative of narcotic activity when tested at concentrations up to 1.0 M. Only ethanol was observed to produce narcotic activity with an IC_{50} of 261 mM (Table 2). No evidence of an IC_{50} by time effect was noted for the solvent test materials. Locomotor activity assessed at 6 dpf was not affected by solvent exposure, nor was evidence of toxicity observed for any of the solvents up to the highest concentrations tested.

Table 2
IC_{50} and TC_{50} values of surfactant test materials tested at 5 dpf.

Test Material	IC_{50} Estimate, μM (95% Confidence Interval)	$t_{1/2}$ Estimate, Sec (95% Confidence Interval)	TC_{50} Estimate, μM (95% Confidence Interval)	Concentration Range, μM
C2-OH	260951 (200114—316030)	No IC_{50} by time effects observed	> 700000 (a)	69405—700000
C6-OH	202.3 (111.6—294.6)	No IC_{50} by time effects observed	> 2000 (a)	19.83—2000
C7-OH	38.3 (27.6—45.5)	No IC_{50} by time effects observed	> 400 (a)	3.97—400
C8-OH	33.8 (31.6—37.4)	No IC_{50} by time effects observed	> 200 (a)	19.83—200
C9-OH	16.3 (14.0—19.9)	No IC_{50} by time effects observed	> 90 (a)	8.92—90
C10-OH	12.7 (11.2—13.9)	140 (17—564)	> 50 (a)	4.96—50
C11-OH	11.3 (2.6E-07—12.9)	124 (18—2458)	> 50 (a)	4.96—50
C12-OH	9.23 (2.05E-08—11.71)	83 (57—255)	> 100 (a)	0.99—100
C13-OH	N/A	N/A	N/A	0.02—10
C14-OH	N/A	N/A	N/A	0.02—10
C15-OH	N/A	N/A	N/A	0.002—1
C16-OH	N/A	N/A	N/A	0.002—1
Glycerol	> 1000000	No response by time effects observed	> 1000000 (a)	34.57—1000000
1,2-Propanediol	> 1000000	No response by time effects observed	> 1000000 (a)	34.57—1000000
C12-EO9	2.24 (1.06—2.60)	427 (308—628)	> 25 (a)	0.25—25
C12-16-EO7	2.36 (2.06—2.65)	332 (274—395)	16.98 (14.38—20.81)	0.25—25
C12-14-EO9	2.12 (1.81—2.40)	364 (278—486)	17.02 (13.32—25.09)	0.25—25
C14-15-EO7	2.12 (1.74—2.65)	337 (258—420)	8.45 (7.23—10.02)	0.25—25
C10-13-LAS	36.7 (33.42—37.34)	175 (24—652)	37.75 (32.92—43.82)	11.90—120
C11-15-AE3S	49.2 (7.7E-08—61.52)	1049 (443—4253)	70.43 (62.99—79.35)	19.83—200
Etomidate	0.39 (0.10—0.86)	No IC_{50} by time effects observed	> 40 (a)	0.01—40

a. No toxicity observed up to the highest concentration tested.

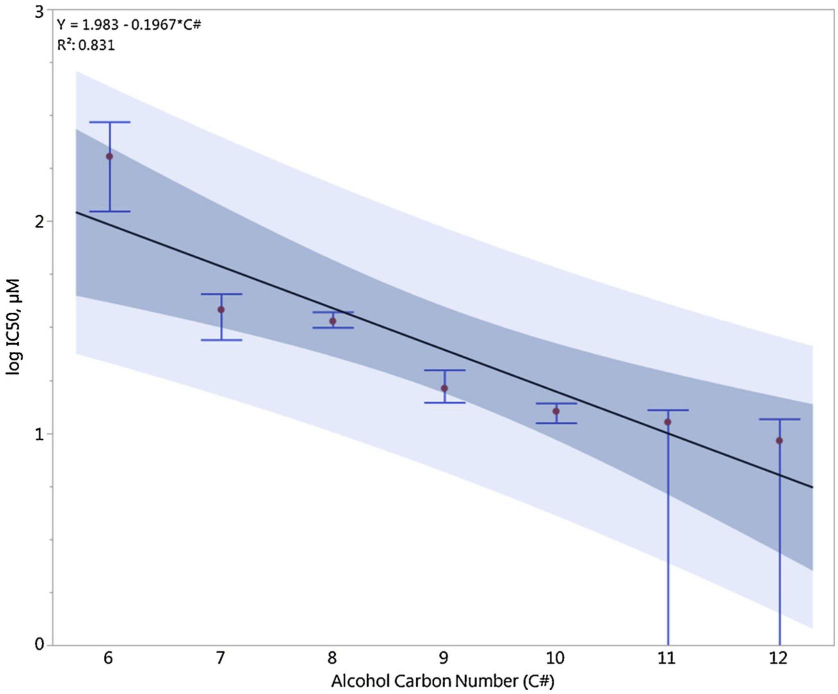

Fig. 2. Narcosis effects for a series of linear, primary alcohols. For a series of 7 linear, primary alcohols, the narcotic potential was correlated with the carbon number of the alcohol. As carbon number increased, IC_{50} for narcosis decreased. Error bars represent the 95% confidence interval for the IC_{50} estimate of each alcohol (see Table 2 for values). The dark shaded area represents the 95% confidence region for the fitted line. The light shaded area represents the 95% confidence region for individual predicted values.

3.3. Surfactants

A series of technical grade surfactants were assessed for narcotic potency in the larval zebrafish neurobehavioral assay (Table 2). Four alcohol ethoxylate test materials were evaluated, as these types of surfactants had been shown previously to have narcotic and anesthetic properties (Talmage, 1994; Zerkle et al., 1987). Two examples of anionic surfactants were also tested, an alcohol ethoxy sulfate (C11-15-AE3S) and a linear alkylbenzene sulfonate (C10-13-LAS). Although not known to possess narcotic activity, anionic surfactants of this type are commonly used surfactants in cleaning products. All of the surfactants except for the C12-EO9 material contain a range of alkyl chain lengths as specified in the test material designation (e.g., C12-16-EO7 contains a range of alkyl chain lengths from C12 to C16). For the alcohol ethoxylate materials, the degree of molar substitution of ethylene oxide is an average (e.g., C12-EO9 has an average molar substitution of ethylene oxide of 9 mol).

All surfactants tested exhibited a suppression of locomotor activity when evaluated at 5 dpf. The IC_{50} values for the surfactants are provided in Table 2. The alcohol ethoxylate test materials evaluated were 17- to 23-fold more potent narcotic agents than the anionic surfactants tested (C10-13-LAS and C11-15-AE3S). Except for the C12-EO9 material, all of the surfactants also were acutely toxic to the zebrafish larvae (Fig. 3 and Table 2). In general, the TC_{50} values for the alcohol ethoxylate test materials were 4- to 8-fold greater than the narcosis IC_{50} values. Thus, although narcosis and toxicity both reside on the concentration-effect curves, these data indicate narcosis and toxicity can be separated for the alcohol ethoxylates. For the anionic surfactants, the IC_{50} values for narcosis and the TC_{50} values for toxicity were approximately equivalent for the C10-13-LAS material and 1.4-fold different for the C11-15-AE3S material. Consequently, the changes in locomotor activity produced by these chemicals was likely attributable to toxicity and not a specific narcotic effect.

For all surfactant test materials, surviving larvae reassessed in the neurobehavioral assay at 6 dpf did not exhibit any significant differences in locomotor activity when compared to control larvae. Thus, neither the anionic surfactants nor the alcohol ethoxylate surfactants produced persistent locomotor effects in surviving larvae.

All of the alcohol ethoxylate materials exhibited an IC_{50} by time effect. The $t_{1/2}$ values for the surfactants are provided in Table 2. This effect is illustrated for the C12-EO9 material which was typical of the alcohol ethoxylates (Fig. 4). The primary purpose for assessing the IC_{50} by time effect is to ensure that the assay continues for a sufficient duration to establish an accurate IC_{50} value for a test material.

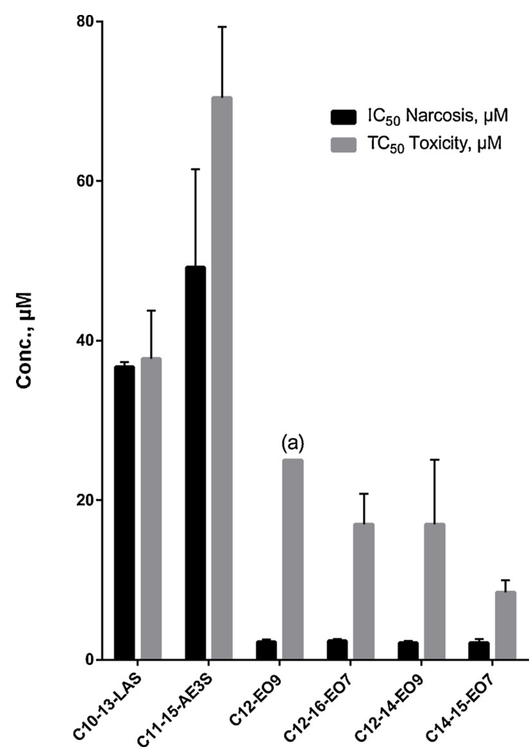

Fig. 3. Surfactant narcosis and toxicity potential. A comparison of the narcosis and toxicity potential of six surfactants. The C12-E09 surfactant (a) did not cause any morbidity or mortality up to the highest concentration tested (25 µM). All other surfactants tested produced both narcosis and toxicity over the concentration ranges evaluated. The error bars represent the 95% confidence intervals.

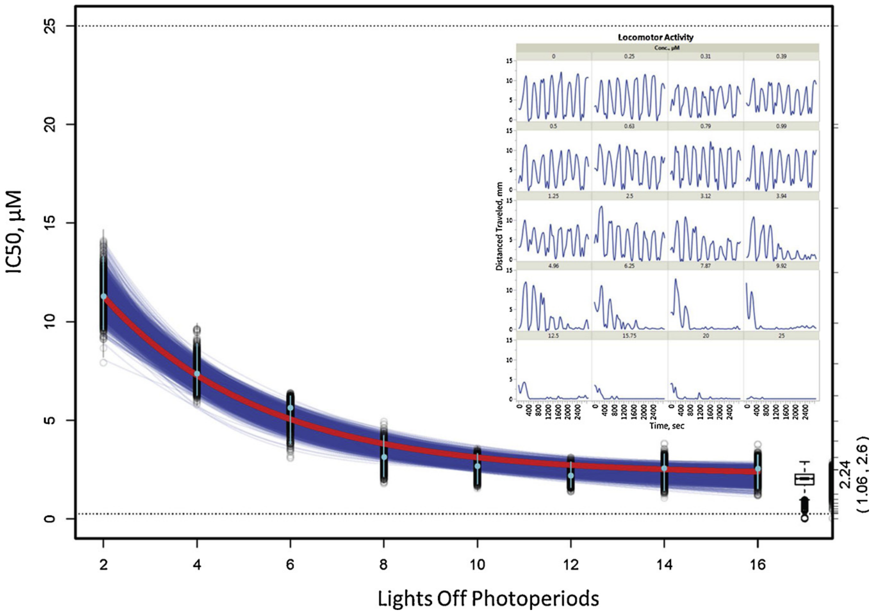

Fig. 4. IC_{50} by time curve for C12-EO9 with exponential decay analysis. IC_{50} values were estimated for each dark photoperiod and then analyzed using an exponential decay approach (red line). A final IC_{50} was determined for the experiment, as well as the half-time of the activity decay curve ($t_{½}$) where statistical bootstrapping (blue lines) was used to quantify uncertainty in the final parameter estimates. The inset shows the raw activity by time curves for the experiment. Upper and lower dotted lines represent the high and low concentrations of test material. The values on the right margin are the final IC_{50} value and the 95% confidence interval. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

However, the $t_{1/2}$ values may also offer insight into toxicokinetic effects for test materials in the larval zebrafish neurobehavioral assay. For example, while ethoxylation may serve to increase the narcotic potency of an alcoholic test material, ethoxylation may also impart properties that delay the uptake of a test material such that it has a delayed narcosis effect. Thus, the linear primary alcohols exhibited little or no IC_{50} by time effect, whereas the corresponding alcohol ethoxylates had a significant delay in the full onset of narcosis.

3.4. Case study demonstrating zebrafish narcosis using known human general anesthetic

Etomidate, an anesthetic agent with known effects in humans (Chau, 2010; DailyMed, 2017), was selected to serve as a reference material for comparing the narcotic potencies of test materials assessed in the larval zebrafish neurobehavioral assay. The IC_{50} for etomidate was determined to be 0.38 µM, the lowest IC_{50} of any of the 21 materials tested. Etomidate narcosis was rapid in onset; no IC_{50} by time effect was noted. No toxicity was observed for etomidate when tested up to a concentration of 40 µM. However, following test material removal, concentration-dependent suppression of locomotor activity was observed at 6 dpf for the higher concentrations of etomidate evaluated (20 µM or greater) indicating that this test material can elicit persistent effects at higher concentrations (i.e., concentrations 50-fold higher than the IC_{50}).

4. Discussion

These studies demonstrate that the larval zebrafish neurobehavioral method can be used to assess narcotic effects and, by incorporating phenotypic screening and recovery assessment steps, determine if such effects are influenced by toxicity. Importantly, this provides a means to better understand if the concentration-dependent reduction in locomotor activity associated with a test material is due to narcosis, toxicity, or a combination. Removing test material via serial dilution and replacement of embryo media allows for a determination as to whether the observed narcotic effects are reversible. Reassessment of locomotor activity in the larvae at 6 dpf following test material removal demonstrated that the narcotic effects of the test materials were indeed reversible. The major limitation of the method is that chemicals must be sufficiently soluble in embryo media to produce effects on locomotor activity. The C12-OH material is either approaching or at the water solubility limit of test materials that can be assessed.

The larval zebrafish neurobehavioral assay can detect and characterize the narcotic activity of a variety of chemicals including linear primary alcohols, solvents, surfactants, and a pharmacological agent, etomidate, with known anesthetic properties in humans (Chau, 2010; DailyMed, 2017). The method is also capable of detecting differences in narcotic potency based on chemical structure. Potential structure-activity relationships were noted across the series of materials evaluated. Aside from the carbon number-narcotic potency relationship observed for the alcohols, it was noted that ethoxylation may increase narcotic potency of alcohols (C12-OH vs. C12-EO9). In addition, the sulfation of an alcohol ethoxylate (C11-15-AE3S) may reduce or eliminate narcotic potency. Note that analytical measures of chemical uptake rates and tissue concentrations were not made in these studies. This data would be needed to confirm that the observed relationships between narcotic potency and chemical structure are not due pharmacokinetic differences between the materials tested. Nevertheless, the larval zebrafish neurobehavioral method offers the ability to investigate structure-activity relationships for chemicals with narcotic activity.

Two chemicals with significant human exposure were included in the set of chemicals evaluated, ethanol and etomidate. The narcosis IC_{50} values determined for ethanol and etomidate were 261 mM and 0.39 µM, respectively. For comparison, the blood ethanol concentration at which approximately 50% of humans are grossly intoxicated is reported as 32.6 mM (150 mg/dL) (Fleming et al., 2005). The plasma level of etomidate determined to produce hypnosis in humans is reported as 0.94 µM (0.23 µg/mL) (Giese and Stanley, 1983). The values reported from humans are for different endpoints on the hypnosis-anesthesia spectrum. Intoxication is a condition which there is impairment, but no loss of responsiveness to environmental stimuli. Conversely, hypnosis is a sleep-like state in which the ability to respond to environmental is impaired or lost. Thus, considering the differences in endpoints, there appears to be at least a general agreement between the IC_{50} values from larval zebrafish and the human values. Nevertheless, additional studies would be needed to establish a relationship between narcosis IC_{50} values derived from larval zebrafish and human plasma concentrations associated with intoxication, hypnosis, or general anesthesia.

Narcosis produced by anesthetic compounds has been evaluated using a variety of techniques ranging from high throughput in vitro screening up through animal testing (McKinstry-Wu et al., 2015). A prime area of interest is the concern related to long term consequences (i.e., neurotoxicity) of anesthetics, particularly in children, which is an active area of research (Lin et al., 2017). As noted, the larval zebrafish is an attractive model because it can be used as a high throughput assay based on behavioral effects and can be investigated on a systems basis down to molecular events. Moreover, the larval zebrafish display complex behaviors which are altered by pharmacological agents in a conserved manner (Rihel et al., 2010). For example, studies have shown that larval zebrafish are responsive to CNS drugs including a chemically diverse class of sedative/hypnotic agents (Renier et al., 2007) and ethanol (Guo et al., 2015). Thus, for identifying novel compounds with anesthetic potential, larval zebrafish may prove a useful model. This is also true for chemicals which are not intended as pharmaceuticals but rather are commonplace in everyday household products.

5. Conclusion

Taken together, the data presented show that a neurobehavioral assay using larval zebrafish provides a robust method to investigate the concentration-dependent narcotic properties of chemicals. Although the focus of these studies has been on chemicals used in household cleaning products, the method is anticipated to be broadly applicable to a wide range of chemicals including pharmaceutical agents and anesthetic/hypnotic agents. Because zebrafish as an organism are readily amenable to genetic manipulation, it is anticipated that genetically modified strains can be developed with altered responses to chemical narcosis. In this context, the neurobehavioral assay presented may be useful for gaining a greater mechanistic understanding of the underlying biology of narcosis and anesthesia.

Competing financial interests

The authors declare they have no actual or potential competing financial interests.

Acknowledgements

This work was supported by The Procter & Gamble Company and NIEHS grant number P30 ES000210.

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