Toxicology Reports 3 (2016) 572–583

Contents lists available at ScienceDirect

Toxicology Reports

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

Evaluation of time-dependent toxicity and combined effects for a series of mono-halogenated acetonitrile-containing binary mixtures

Douglas A. Dawson[a,*], Daphne Guinn[a], Gerald Pöch[b]

[a] Department of Biology/Toxicology, Ashland University, Ashland, OH, USA
[b] Department of Pharmacology and Toxicology, University of Graz, Graz, Austria

ARTICLE INFO

Article history:
Received 14 June 2016
Received in revised form 22 July 2016
Accepted 22 July 2016
Available online 25 July 2016

Chemical compounds studied in this article:
Iodoacetonitrile (PubChem CID: 69356)
Bromoacetonitrile (PubChem CID: 11534)
Chloroacetonitrile (PubChem CID: 7856)
Dibromoacetonitrile (PubChem CID: 18617)
Ethyl acrylate (PubChem CID: 8821)
Ethyl bromoacetate (PubChem CID: 7748)
Ethyl propiolate (PubChem CID: 12182)
Linalool (PubChem CID: 6549)
Methyl vinyl ketone (PubChem CID: 6570)
Trichloroacetonitrile (PubChem CID: 11011)

Keywords:
Microtox®
Acute toxicity
Dose-addition
Independence

ABSTRACT

Mixture and time-dependent toxicity (TDT) was assessed for a series of mono-halogenated acetonitrile-containing combinations. Inhibition of bioluminescence in *Aliivibrio fischeri* was measured after 15, 30 and 45-min of exposure. Concentration-response (x/y) curves were determined for each chemical alone at each timepoint, and used to develop predicted x/y curves for the dose-addition and independence models of combined effect. The x/y data for each binary mixture was then evaluated against the predicted mixture curves. Two metrics of mixture toxicity were calculated per combined effect model: (1) an EC_{50}-based dose-addition (AQ) or independence (IQ) quotient and (2) the mixture/dose-addition (MX/DA) and mixture/independence (MX/I) metrics. For each single chemical and mixture tested, TDT was also calculated. After 45-min of exposure, 25 of 67 mixtures produced curves that were consistent with dose-addition using the MX/DA metric, with the other 42 being less toxic than predicted by MX/DA. Some mixtures had toxicity that was consistent with both dose-addition and independence. In general, those that were less toxic than predicted for dose-addition were also less toxic than predicted for independence. Of the 25 combinations that were consistent with dose-addition, 22 (88%) mixtures contained chemicals for which the individual TDT values were both >80%. In contrast, of the 42 non-dose-additive combinations, only 2 (4.8%) of the mixtures had both chemicals with individual TDT values >80%. The results support previous findings that TDT determinations can be useful for predicting chemical mixture toxicity.

1. Introduction

Determining and predicting the effects of toxic substances in combination has been a focal point of recent mixture toxicity research. Via exposure profiling of reactive mixtures [1], docking-based receptor library [2] or toxicogenomics [3] studies, or examining aquatic toxicity [4], mammalian reproductive effects [5] or endocrine disruptors [6] such research efforts can improve the ability to predict mixture toxicity. Assessments of toxicity in binary [7], ternary [8] or complex chemical mixtures [9] have been common, with such toxicity having been evaluated for metals [10], pesticides [11], polycyclic aromatic hydrocarbons [12,13], and micropollutants [14]. Conceptual studies and efforts to develop and evaluate mixture toxicity models [15–20] have also provided approaches for improving toxicity assessment of mixtures.

Microtox® is one assay often used to examine chemical mixture toxicity. This system makes use of bacteria that, through the process of quorum-sensing, produce light that can be reliably quantified by a light meter. When the bacteria are exposed to single chemicals or chemical mixtures at concentrations that exert toxicity, metabolism is adversely affected, thereby reducing the amount of light emitted. Light readings can be made prior to chemical exposure and for up to three selected timepoints after introduction of the toxicant. For the acute toxicity assay exposure can last for up to 100 min; after that point bacterial metabolism begins to wane as no nutrients are included in the reagent. Since readings can be made after chemical exposure at three selected timepoints, it is possible to assess the time-dependent toxicity of a given chemical, mixture, or environmental sample. Agents that act as non-polar narcotics tend to show inhibition of bioluminescence early on during expo-

* Corresponding author.
E-mail address: ddawson2@ashland.edu (D.A. Dawson).

http://dx.doi.org/10.1016/j.toxrep.2016.07.003

sure but then recover, at least partially, so that bioluminescence stabilizes or increases slightly after the initial diminution. For other chemicals toxicity continues to progress over exposure time such that bioluminescence decreases throughout exposure. These features allow the assay to be used effectively for assessing changes in toxicity over exposure time (i.e., time-dependent toxicity).

Recent studies from this lab have evaluated time-dependent toxicity (TDT) for potential value in mixture toxicity prediction using Microtox®. Initial studies examined mixture toxicity for binary combinations of soft electrophiles [21]; subsequently examining such toxicity in the context of chemical reactivity [22,23]. Through binary mixture assessments of S_N2-reactive α-halogenated acetonitriles [24], ethyl α-halogenated ethyl acetates [25], and combinations of these two groups [26] it was determined that including TDT assessments added value to mixture toxicity studies. An asymmetry parameter was incorporated into curve-fitting of single-chemical and mixture concentration-response (x/y) data to more precisely evaluate mixture toxicity against the dose-addition and independence models of combined effect [27]. Most recently TDT assessments were examined for use in predicting mixture toxicity [28].

In the latter study, it was demonstrated that taking the average TDT values of the individual chemicals in a mixture could be used to predict the TDT of the mixture [28]. This was true even when chemicals with high TDT (i.e., ≥90%) were tested with ones having low (i.e., <30%) or negative TDT despite the observed mixture TDT value being more likely to deviate from the predicted TDT value. Having previously incorporated the asymmetry parameter into curve-fitting of x/y data it became of interest to specifically assess aspects of the curve-fitting parameters for insights into the relationship between TDT and combined effect.

As noted above, α-halogenated acetonitriles are S_N2-reactive soft electrophiles. In previous studies from this lab three mono-halogenated acetonitriles (i.e., iodoacetonitrile – IAN, bromoacetonitrile – BRAN and chloroacetonitrile – CLAN) were tested in sham combinations and with each other [24], with ethyl α-halogenated ethyl acetates [26], and with a few other organic chemicals [28]. The latter study only reported TDT values for the single chemicals and mixtures, not the specific combined effects observed. In this study, the three mono-halogenated acetonitriles (XANs) were tested in binary combination with a number of additional organic chemicals. The latter were selected to span the range of TDT values (<0%–>100%) and were compiled along with the previous XANs mixture data to fully evaluate combined effects as they relate to TDT. In order to provide a consistent basis for this assessment, the maximum effect constraint in curve-fitting was fixed at 100%. A collective summary of these results, including data for 28 combinations previously unpublished in any form, is provided herein.

2. Materials and methods

2.1. Chemicals and reagents

Chemicals used in testing (Table 1) were obtained from Sigma-Aldrich (Milwaukee, WI) at ≥95% purity and used as received. Microtox® bacterial reagent, reconstitution solution and diluent were obtained from Modern Water, Inc. (New Castle, DE).

2.2. Toxicity testing

A routinely calibrated Microtox® 500 analyzer was used to determine inhibition of bioluminescence in the marine bacterium *Aliivibrio fischeri* (formerly *Vibrio fischeri*) [29] following established procedures [26]. An experiment in this testing protocol is defined as consisting of three toxicity tests: chemical A-alone (A), chemical B-alone (B) and a "true" mixture (A + B). Some experiments were of the "sham" variety, in which two preparations of a chemical were tested alone (i.e., A_1 and A_2) and combined as a "mixture" (i.e., $A_1 + A_2$).

Table 1
Chemicals selected for testing.

Abbr.	Chemical name	CAS #[a]
3M2B	3-methyl-2-butanone	563-80-4
4NBB	4-nitrobenzyl bromide	100-11-8
BGE	butyl glycidyl ether	2426-08-6
BRAN	bromoacetonitrile	590-17-0
CLAN	chloroacetonitrile	107-14-2
DBRAN	dibromoacetonitrile	3252-43-5
DCLAN	dichloroacetonitrile	3018-12-0
DEM	diethyl maleate	141-05-9
EA	ethyl acrylate	140-88-5
EAC	ethyl acetate	141-78-6
EBAC	ethyl bromoacetate	105-36-2
ECAC	ethyl chloroacetate	105-39-5
EFAC	ethyl fluoroacetate	459-72-3
EIAC	ethyl iodoacetate	623-48-3
EP	ethyl propiolate	623-47-2
IAN	iodoacetonitrile	624-75-9
LIN	linalool	78-70-6
M2BP	methyl-2-bromopropionate	5445-17-0
MC	methyl crotonate	623-43-8
MVK	methyl vinyl ketone	78-94-4
NER	nerol	106–25-2
PN	propionitrile	107–12-0
TCLAN	trichloroacetonitrile	545–06-2

[a] Chemical Abstract Service registry number.

Concentration selection for each chemical was made based on results of preliminary tests and, as much as possible, designed to obtain an approximately equitoxic potency ratio (i.e., 1:1) after 30-min of exposure. At least seven concentrations were tested in duplicate (i.e., two vials per concentration) for each chemical or mixture along with a duplicated control. Nominal concentrations, corrected for density, were prepared via serial dilution. For any given experiment a single dilution factor (1.6, 1.75, 1.867, or 2.0) was used, having been selected to most effectively calculate EC_{25}, EC_{50} and EC_{75} values, based on preliminary testing. The EC_{50} refers to the half-maximal effective concentration, while the EC_{25} and EC_{75} represent the one-quarter and three-quarters-maximal effective concentrations, respectively.

For each experiment, chemical A, chemical B and the mixture of A and B were tested on the same day, typically within a 4.5 h time period. Separate stock solutions of chemical A and chemical B were prepared immediately prior to testing. The mixture stock solution was prepared from the single chemical stock solutions. In testing, initial light readings were taken before chemical exposure. During exposure light readings were taken 15, 30 and 45-min after toxicant introduction. Microtox® Omni software calculated the percent effect value for each vial at each exposure duration.

2.3. Curve fitting

Nine concentration-response (x/y) curves (i.e., three curves for chemical A: one each at 15, 30 and 45-min, along with three curves each for chemical B and the mixture at those same timepoints) were obtained from each experiment. After input to SigmaPlot® (v. 11.0; Systat Software, Chicago, IL) x/y data were fitted to sigmoid curves using the 5-parameter logistic minus 1-parameter (5PL-1P) function [27]. This approach utilized four parameters: EC_{50}, slope, maximum effect and asymmetry, as the minimum effect parameter had been removed from the original 5PL function within the software.