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Mixture Toxicity of S_N2-Reactive Soft Electrophiles: 1. Evaluation of Mixtures Containing α -Halogenated Acetonitriles

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Abstract

The concept of multiple modes of toxic action denotes that an individual chemical can induce two or more toxic effects within the same series of concentrations, for example, reactive toxicity and narcosis. It appears that such toxicity confounds the ability to develop precise predictions of mixture toxicity and makes it more difficult to clearly link a dose-additive combined effect to agents in the mixture having a single common mechanism of toxic action. This initial study of a three-part series begins to examine this issue in greater detail by testing three α -halogenated acetonitriles: (1) in sham combinations, (2) in true combinations, and (3) with a nonreactive nonpolar narcotic. Iodo-, bromo-, and chloro-derivatives of acetonitrile were selected for testing based on their electro(nucleo)philic reactivity, via the S_N2 mechanism, and their time-dependent toxicity individually. Reactivity of each agent was assessed in tests with the model nucleophile glutathione (GSH). Each acetonitrile was reactive with GSH, but the nonpolar narcotic 3-methyl-2-butanone was not. In addition, toxicity of the agents alone and in mixtures was assessed using the Microtox[®] acute toxicity test at three time points: 15, 30, and 45 min of exposure. Each of the three agents alone had time-dependent toxicity values of about 100%, making it likely that most of the toxicity of these agents, at these times, was due to reactivity. In contrast, the nonpolar narcotic agent lacked time-dependent toxicity. In mixture testing, sham combinations of each acetonitrile showed a combined effect consistent with predicted effects for dose-addition at each time point, as did the sham combination of the nonpolar narcotic. Mixture toxicity results for true acetonitrile combinations were also consistent with dose-addition, but the acetonitrile–nonpolar narcotic combinations were generally not consistent with either the dose-addition or independence models of combined effect. Based on current understanding of mixture toxicity, these results were expected and provide a foundation for the second and third studies in the series.

Understanding chemical mixture toxicity on the basis of a mode or mechanism of toxic action is one goal of mixture toxicity research (Borgert et al. 2004; Broderius et al. 1995; Escher and

Hermens 2002; Pösch 1993). The primary purpose of such works is to be able to predict the combined effect based on the toxic mode(s) or mechanism(s) of the chemicals. The ability to do so would greatly reduce the number of mixture toxicity tests needed to help ensure public health or ecosystem safety.

In such research, two predictive models are often used to assess the combined effect of a mixture: dose-addition (e.g., Chen et al. 2001) and independence (Bliss 1939). These models have been described as being mechanistic models (Dawson et al. 2002; Pösch et al. 1990, 1996) since the former occurs, theoretically, when the two agents are toxic by the same, single mechanism of action, whereas the latter occurs when the toxicity of the first agent has no effect on the toxicity of the second agent (and vice versa), such that the agents are likely to have distinct mechanisms of action.

Predicting mixture toxicity on the basis of a mode or mechanism of toxic action can be difficult (Borgert et al. 2004). One reason for this is that many mixtures of organic chemicals show toxicity that is approximately additive (Calabrese 1991; Rand et al. 1995), as noted by results of numerous studies (e.g., Deneer et al. 1988; Hermens et al. 1984, 1985). This, in part, led to the suggestion (Hodges et al. 2006; McCarty and Mackay 1993; Rand et al. 1995) that some toxic chemicals will exert baseline toxicity (i.e., narcosis) at concentrations close to or the same as those that cause toxicity by another mode of action, such as through reactivity with biological nucleophiles. Recent studies using Microtox[®] to examine mixture toxicity of soft electrophiles have generated data that support this concept of multiple modes of toxic action (Dawson et al. 2008; Gagan et al. 2007).

Reactive chemicals have toxicity that increases over time in Microtox[®] (Dawson et al. 2006, 2008; Gagan et al. 2007). When the toxicity of an agent (or mixture) is determined at multiple time points during a test, the change in toxicity over time (E) can be readily calculated based on the well-known equation $E = c \times t$, where c is concentration and t is time; for example, an agent with an EC₅₀ after 30 min that is half of that at 15 min has a time-dependent toxicity (TDT) value of 100% for that time period (Gagan et al. 2007). Based on the results of these earlier studies, it has been suggested that agents having TDT values of 100% or more exert toxicity almost exclusively through reactivity with cellular nucleophiles. In comparison, agents with TDT values between about 10% and 90% are thought to induce toxicity via both reactivity and narcosis (Dawson et al. 2008; Gagan et al. 2007), with reactive toxicity being more prominent at higher TDT values. Nonpolar narcotic agents have little or no time-dependent increase in toxicity and often have negative TDT values, most likely due to recovery from toxic effects and toxicant elimination.

As a result of these findings, time-dependent toxicity assessment has been included as an important component of mixture toxicity assessment (Dawson et al. 2008; Gagan et al. 2007). In these studies, soft electrophiles reactive by Michael-type, S_N2, and S_NAr mechanisms were evaluated for toxicity both singly and in binary mixtures with either another soft electrophile or a model nonpolar narcotic. For the latter combinations, combined effects that were approximately, but not clearly, dose-additive were frequently observed.

Based on these works, a series of three studies has been initiated to examine more fully the relationship between mode/mechanism of toxic action and chemical mixture toxicity. These studies were to include examination of chemical reactivity, as well as calculation of time-dependent toxicity. In the initial study in this series, reported herein, three α -halogenated acetonitriles, each having TDT values of about 100% at each time point and being reactive by the S_N2 mechanism, were tested both singly and in binary combinations with each other and with a nonreactive, nonpolar narcotic, in order to evaluate mixture toxicity and the concept of multiple modes of toxic action. The hypothesis for the study was that these three α -halogenated

acetonitriles, (1) being structurally similar, (2) being S_N2 -reactive with the model nucleophile glutathione, and (3) having about 100% time-dependent toxicity, would have the same single mechanism of toxicity and show combined effects consistent with dose-addition. However, when each agent was tested with the nonpolar narcotic, the combined effect would not be consistent with dose-addition, as the modes of toxic action of the agents would be distinct, i.e., reactivity for the acetonitriles and narcosis for the nonpolar narcotic.

Materials and Methods

Chemicals

Chemicals tested were (abbreviation, Chemical Abstract Service registry number, purity): iodoacetonitrile (IAN, 624-75-9, 98%), bromoacetonitrile (BRAN, 590-17-0, 97%), chloroacetonitrile (CLAN, 107-14-2, 99%), and 3-methyl-2-butanone (3M2B, 563-80-4, 99%). Each was purchased from Sigma-Aldrich and not further purified. Stock solutions of each agent were prepared by dissolution in Microtox[®] diluent (a manufacturer-prepared and purified 2% NaCl solution) or in dimethyl sulfoxide (DMSO, maximum concentration in testing 0.1%) with subsequent dilution in the diluent. Stock solutions were freshly prepared just prior to testing and held in the dark at 15°C in tightly closed glass vials.

In Chemico Reactivity Assessment With GSH

To quantify chemical reactivity experimentally, the thiol group of the tripeptide glutathione (GSH) was used as described by Schultz et al. (2005). During a 2-h incubation period *in chemico*, in a concentration–response manner, free thiol was quantified spectrophotometrically at 412 nm by its reaction with the chromophore 5,5'-dithio-bis(2)-nitrobenzoic acid. The 50% reactive concentrations (RC_{50} values in mM, mean of two tests) were determined from nominal concentrations of the toxicants (IAN, BRAN, CLAN, and 3M2B) using the probit analysis procedure of Statistical Analysis Systems software.

Toxicity Experiments

Microtox[®] (Strategic Diagnostics, Inc., Newark, DE) acute toxicity testing procedures were used, with inhibition of bioluminescence serving as the toxicity endpoint. For each chemical combination there was a test of each agent alone (agent A, agent B) and a mixture of the two agents (A–B). Each test had seven duplicated concentrations and a duplicated control treatment. All concentrations were nominal and density corrected. Test concentrations were prepared by serial dilution using 1.867 as the dilution factor. A sham combination of each agent (e.g., IAN–IAN) was also tested using the same experimental design. For CLAN, two sham tests were conducted using different relative potency ratios as a means of evaluating consistency of the combined effect.

The freeze-dried bacterial reagent (*Vibrio fischeri*) was reconstituted 15–20 min prior to test initiation and held at $5 \pm 0.1^\circ\text{C}$. Initial readings for each treatment vial quantified bioluminescence without chemical. Once toxicant solution was added, readings were taken at 15, 30, and 45 min of exposure. Treatment vials were held at $15 \pm 0.2^\circ\text{C}$ throughout testing with the pH of the solutions ranging from 7.2 to 7.7.

Statistical Methods for Single Chemical and Mixture Toxicity Data

Percentage effect data (inhibition of bioluminescence) for the single agents and mixtures were obtained using MicrotoxOmni[®] software. Toxicity data were transferred to SigmaPlot version 11.0 (Systat Software, Chicago, IL) for use in custom-made worksheets and program files. Experimental data points for each single agent and mixture test were fit using sigmoid curves using a four-parameter logistic function (Dawson et al. 2006; Pösch, 1993). The following

constraints were used for fitting the data to sigmoid curves: $0 < \text{minimum effect} < 1$; maximum effect $< 100\%$. The slope and EC_{50} values for each single agent and mixture test were calculated at each time point (15, 30, and 45 min) along with minimum and maximum effect parameters and the correlation coefficient (r^2) for the concentration–response data, with inclusion of all data points. The data were then used to calculate time-dependent toxicity (TDT) values for each agent as described previously (Gagan et al. 2007).

For mixture data, total chemical concentrations were expressed in terms of concentration equivalents of the more toxic agent in the combination (i.e., conversion factor for chemical B = concentration of chemical A/concentration of chemical B) as described previously (Dawson et al. 2002). Experimental mixture EC_{25} , EC_{50} , EC_{75} , and slope values were calculated and converted to additivity quotient (AQ) values, which are similar to toxic unit values often calculated in mixture toxicity studies (Dawson et al. 2008). Herein, AQ values were calculated as $AQ = \text{experimental value/predicted value for dose-addition}$. The predicted dose-addition value was obtained from the theoretical dose-addition concentration–response curve, which had been calculated from the single chemical data as described previously (Dawson et al. 2000, 2004; Pösch et al. 1990). Theoretical curves for the independence model of combined effect were also developed for visual comparison with theoretical dose-addition curves (Gagan et al. 2007). Independence quotient (IQ) values for the EC_{25} , EC_{50} , and EC_{75} data were also calculated, with $IQ = \text{experimental value/predicted value for independence}$.

With the differing time-dependent toxicities of the agents, a relative potency ratio was calculated for each mixture at each time point. Relative potency ratio values show the deviation from an equieffective (i.e., 1:1) concentration ratio of the two agents in the mixture at a given exposure time. Changes in the relative potency ratios for a combination are the result of different levels of time-dependent toxicity for each single agent. For example, at 15 min, agent A could have lower toxic potency than agent B, giving, for example, a 1.0:1.2 relative potency ratio (i.e., B was more potent), but due to the greater time-dependent toxicity for agent A the ratio might become a 1.0:1.0 at 30 min and 1.0:0.8 at 45 min.

Quality of the concentration–response data was examined in two ways. The first was via calculation of correlation coefficients (r^2) for each single chemical and mixture curve. The second approach evaluated test-to-test consistency of each agent alone by calculating coefficient of variation (CV) values for the EC_{50} and slope parameters at each time point. It has been noted that reporting CV, rather than standard error, values is often preferable when data result from work carried out by multiple operators (Steel and Torrie 1980).

Results

Single Chemical Data

Each of the four chemicals tested in the study were evaluated individually for reactivity with the model nucleophile glutathione (GSH) and for toxicity in Microtox[®] (Table 1). The α -halogenated acetonitriles showed reactivity with GSH, with the order of relative reactivity being: $IAN \approx BRAN > CLAN$ (Table 1, column 2), consistent with previous work (Hine 1962). Relative toxicity of these three agents showed the same pattern, consistent with previous work (Roberts et al. 2010). In addition, for each of these three agents, time-dependent toxicity was about 100% at each testing interval (Table 1, columns 9–11). In contrast, 3-methyl-2-butanone, a nonpolar narcotic, lacked reactivity and showed toxicity that decreased slightly over time (Table 1, columns 9–11). Slope values for the toxicity data of the reactive agents singly (IAN, BRAN, and CLAN) were about 1.5 after 15 min of exposure and increased slightly (1.6–1.7) at 30 and 45 min (Table 1, columns 6–8). Slope values of about 1.0 were obtained for the nonpolar narcotic (3M2B) at each time point.

Mixture toxicity: Sham Combinations

Five sham combinations were tested (Table 2, Fig. 1), including CLAN–CLAN twice at different relative potency ratios. For each combination, at each time point, the combined effect was consistent with dose-addition ($EC - AQ = 1.0 \pm 0.1$) at the 25, 50, and 75% effect levels (Table 2, columns 4–6). Slope AQ values were also near 1.0 (Table 2, column 7).

Independence quotient (IQ) values for each sham mixture at each time point showed that the data did not generally fit the independence model of combined effect well (Table 2, columns 8 and 9), with IQ values being below 0.90 at the 25 and 50% effect levels. At the 75% effect level, however, sham data fitted the independence model reasonably well (Table 2, column 10).

Mixture Toxicity: α -Halogenated Acetonitrile Combinations

Three true combinations of α -halogenated acetonitriles were tested (Table 3, Fig. 2). Again, for each combination and time point, EC_{50} –AQ values were close to 1.0 (Table 3, column 5), with those for the BRAN–CLAN combination being furthest from a strict 1.0 value (i.e., 1.05–1.08). Slope–AQ values were also close to 1.0 (Table 3, column 7).

As with the sham mixtures, EC –IQ values for each true mixture, at each time point, showed that the data did not fit the independence model of combined effect well at the 25 and 50% effect levels. Once again, a better fit to the independence model was obtained at the 75% effect level (Table 3, columns 8–10).

Mixture Toxicity: α -Halogenated Acetonitrile, Nonpolar Narcotic Combinations

Each acetonitrile was also tested in combination with the nonpolar narcotic (3M2B) in order to assess the combined effect of the reactive agents with a nonreactive chemical (Table 4, Fig. 3). As judged by EC_{50} –AQ values at each time point, each combination produced mixture toxicity that was less toxic than that predicted for a strict dose-addition (i.e., 1.09–1.30; Table 4, column 5) combined effect. However, at the 25% effect level, AQ values were closer to those typical of dose-addition (0.92–1.07; Table 4, column 4). At the 75% effect level, AQ values were much greater than those expected for dose-addition (1.39–1.58; Table 4, column 6).

In contrast to that for the sham acetonitrile and true acetonitrile mixtures, IQ values for acetonitrile–3M2B mixtures were closer to being consistent with independence at the 25% effect level and, generally, more poorly fitted independence at the 50 and 75% effect levels (Table 4, columns 8–10), with the latter being especially poor (1.20–1.84).

Quality and Consistency of Toxicity Data

The single chemical test data were subjected to two examinations of data quality: (1) by determining correlation coefficient (r^2) values for the fit of concentration–response data to a sigmoid curve, and (2) by calculating the coefficient of variation (CV) for EC_{50} and slope values at each time point. For the former, all single chemical curves ($n = 66$) had r^2 values between 0.9957 and 0.9996 (data not shown), suggesting that seven duplicated concentrations provides sufficient data with which to develop high-quality concentration–response curves. Calculations of test-to-test variation for EC_{50} and slope data, on a chemical-by-chemical basis, resulted in CV values of < 10 at each time point (Table 1). This suggests a high degree of test-to-test consistency for these chemicals, as these CV values are similar to those from previous studies (Dawson et al. 2006,2008;Gagan et al. 2007).

Concentration–response data for the mixtures were also examined for quality by determining r^2 values. All such curves ($n = 33$) had r^2 values between 0.9971 and 0.9999 (data not shown),

again suggesting that seven duplicated concentrations are sufficient for developing high-quality concentration–response data in Microtox[®].

Discussion

Mixture Toxicity and Multiple Modes of Action

Early research on chemical mixture toxicity (e.g., Bliss 1939; Plackett and Hewlett 1952) helped to define the concepts of no interaction and interaction and to describe the types of combined effect that may be encountered (Calabrese 1991). More recently, much work has been done to assess specific hazards of multiple chemical exposures. In such works, dose-addition of nonpolar narcotics has been established (e.g., Broderius and Kahl 1985), and it is generally considered that additivity is a reasonable estimate of mixture toxicity when many chemicals are present in a mixture and each agent is at a low concentration (e.g., Warne and Hawker 1995).

Chemical mixture toxicity studies in aquatic toxicology are sometimes designed to determine modes or mechanisms of toxic action of the chemicals being tested (e.g., Broderius et al. 1995; Dawson et al. 2004; Hodges et al. 2006). Predicting mixture toxicity when only a few chemicals are present and the agents in the mixture have unknown, various, or multiple modes of action is currently of specific interest. One recent study has examined the mixture toxicity of ester sulfonates (Hodges et al. 2006). A key feature of interpreting the mixture toxicity in this study was the consideration that some chemicals do not act exclusively as a nonpolar or a polar narcotic but actually have features of both modes of action (Roberts and Costello, 2003).

The possibility of any given agent having multiple modes of toxic action, for example, reactivity and nonpolar narcosis, within a given range of concentrations needs to be considered in the development of computer models designed to predict chemical mixture toxicity more accurately. This can be exemplified by products that have chemicals added to products to provide fragrance. In such products, a small numbers of chemicals may be used together, with some having reactive toxicity (perhaps by different reaction mechanisms), or one or more of the agents may exert toxicity through multiple modes of action. Since soft electrophiles can be reactive by various mechanisms (Roberts et al. 2007), studies assessing the mixture toxicity of soft electrophiles, both within and across reaction mechanisms, are needed. For these reasons, the current series of mixture studies with S_N2 -reactive soft electrophiles was undertaken.

Electrophiles and Mixture Toxicity

Chemicals that are electron deficient are electrophiles. This electron deficiency allows them to interact with electron-rich chemicals (i.e., nucleophiles) during a reaction. Electrophilic chemicals can be classified as being hard or soft, with this designation being based on the polarizability of the electro(nucleo)philic center of the molecule. When an electrophile reacts with an endogenous nucleophile (most often a hard electrophile reacts with a nucleic acid and a soft electrophile with an amino acid), a covalent bond may form between them. Since living organisms have an abundance of O, N, and S atoms within nucleic acids and amino acids, when an exogenous electrophile gains entry into cells, opportunities for electro(nucleo)philic reactivity to occur are plentiful. When that happens and the concentration of the electrophile within the organism is high enough, such covalent bonding can produce toxicity.

It is of interest to examine such toxicity in chemical mixtures, as the combined toxicity of the agents can be evaluated on a reaction mechanism basis. Common reaction mechanisms for an electrophile and an endogenous nucleophile include addition reactions, such as Michael addition, and various substitution reactions, including the S_N2 , and S_NAr types. In each of the

latter, the leaving group of the electrophilic agent is displaced by a nucleophile on passage through a transition state.

An S_N2 electrophile commonly has a CH_2X group as the reaction center, with X, often a halogen, being the leaving group. If there is an unsaturated carbon atom [e.g., $C=O$, $C(=O)NH_2$, $C\equiv N$] adjacent to the CH_2X group, the reaction is enhanced, thereby enabling a reaction with the soft nucleophilic thiol moiety (Roberts et al. 2010; Schultz et al. 2006, 2007). In the case of the α -halogenated acetonitriles used in this study, the cyano group in the β -position relative to the leaving group stabilizes negative charges, which build up in the transition state of the reaction. This increases the rate of the reaction (Jacobs 1997), so the S_N2 electrophiles tested herein readily react via this replacement mechanism. Additionally, since they also react with agents having a thiol group, such as glutathione, it is reasonable to use that test to measure their relative reactivity (Roberts et al. 2010; Schultz et al. 2006, 2007).

Reactivity of the Agents

The results of the single chemical tests showed that each of the α -halogenated acetonitriles was reactive and had time-dependent toxicity of about 100%. This suggests that most of the toxicity caused by these agents during the timeframe of testing was due to reactivity rather than narcosis. In comparison, the nonpolar narcotic agent alone was not reactive and showed toxicity that diminished slightly between 15 and 45 min of exposure. These results were not unexpected, as they are generally consistent with previous toxicity testing and quantitative structure–activity relationship (QSAR) analyses (Roberts et al. 2010; Schultz et al. 2006, 2007).

Mixture Toxicity and Sham Combinations

Mixture tests in this study included several sham combinations. Sham tests can be quite useful in mixture toxicity studies because an agent inducing toxicity by a single mechanism of action should, conceptually, show dose-addition. Agents having time-dependent toxicity of about 100%, such as these α -halogenated acetonitriles, have the best chance of inducing toxicity by a single mode of action within the concentration range being tested. Likewise, agents lacking an increase in toxicity over time, such as the nonpolar narcotic 3M2B, may also exert toxicity by a single mode of action, although it has been noted that some nonpolar narcotics may act to some degree as polar narcotics in toxicity testing (Hodges et al. 2006), thereby making mixture toxicity predictions more difficult. However, agents that have time-dependent toxicity in the range of about 10–90% appear to have more potential to induce toxicity by multiple modes of toxic action.

Each of the sham combinations of α -halogenated acetonitriles showed a combined toxic effect consistent with the predicted effect for dose-addition. This relationship was observed at the 50% effect level (Table 2, column 5) and at both the 25 and 75% effect levels (Table 2, columns 4 and 6) as well. This result is important because it ensures that the EC_{50} –AQ is not just close to 1.0 by happenstance; for example, it is possible for an EC_{50} –AQ of 1.00 to occur when the predicted curve for dose-addition and the experimental curve happen to cross each other at the 50% effect level, while toxic effects at lower or higher points on the curve are clearly not dose-additive.

Two sham tests using CLAN were conducted in this study: the first at about an equitoxic (i.e., 1:1) relative potency ratio, and the second at a relative potency ratio of about 1.0:0.75 (Table 2, column 3). This was done as a control for the general experimental design, to ensure that a dose-additive combined effect was obtained regardless of the relative potencies of the agents. For agents that induce toxicity by two (or more) modes of action, it is possible that a sham combination may not show dose-additive toxicity, most notably when the relative potency ratio is not close to 1:1. The results for the CLAN–CLAN tests therefore provide a baseline reference

for the true combinations, which might be expected to show dose-addition but were not tested at a strictly equitoxic potency ratio.

When the EC-IQ values for the sham combinations were examined, it was noted that they were typically further from 1.0, indicating that the experimental mixture curves were not consistent with predicted effects for the independence model. This is reasonable since, with independent action, toxicity is thought to be induced through different modes or mechanisms of action.

Mixture Toxicity and True Combinations of Acetonitriles

For the three true combinations of α -halogenated acetonitriles, the combined effect in each case and at each time point was close to that predicted by the dose-addition model and somewhat more toxic than that predicted by the independence model (Table 3, Fig. 2). The experimental data for the IAN-BRAN and IAN-CLAN combinations showed, as judged by EC-AQ values, very good agreement with the predicted curve for dose-addition at each time point, and at the 25%, 50%, and 75% effect levels. This was the case even though the relative potency ratios of the combinations were not always strictly 1:1 (Table 3, column 3).

Mixture Toxicity and Acetonitrile–Nonpolar Narcotic Combinations

The acetonitrile–nonpolar narcotic combinations had EC₅₀-AQ values that were inconsistent with dose-addition (Table 4, column 5), with toxicity being less than that predicted by the model. Although several EC-AQ values at the 25% effect level were close to 1.00 (Table 4, column 4), AQ values at the 75% effect level were all 1.39 or higher, indicating that toxicity at the upper end of the concentration–response curves was less than that predicted for dose-addition. Also, the experimental results for these combinations were not well predicted by the independence model, especially along the middle and upper segments of the concentration–response curves (e.g., Fig. 3).

Since CLAN and 3M2B have similar log *P* values, one might expect that CLAN, despite being reactive, would also produce some toxicity due to narcosis at the concentrations tested. However, early time-course studies using this system showed that CLAN induced toxicity that was time dependent prior to 15 min of exposure (i.e., TDT values of $\geq 90\%$ between 5 and 10 min of exposure and increasing to above 100% between 10 and 15 min of exposure). This demonstrates fairly rapid reactivity for CLAN in *V. fischeri* and suggests that any toxicity due to a narcotic effect of CLAN would be fairly low after 15 min of exposure. It is noted, however, that EC₅₀-AQ values for the CLAN–3M2B combination rose from 1.09 (i.e., closer to dose-additive) at 15 min of exposure to 1.30 after 45 min of exposure. Therefore, the 15-min mixture data reflect the possibility of some narcotic toxicity due to CLAN during the early portion of the test.

There are two other points of note with respect to these acetonitrile–nonpolar narcotic results. First, the relative potency ratios were skewed heavily, by design, toward the nonpolar narcotic (3M2B) at the 15-min time point (e.g., nearly 1.0:4.0 for IAN–3M2B) and moved closer to being equitoxic later in testing. When tested alone, 3M2B had toxicity that was not time dependent, in contrast to that for the acetonitriles, which increased steadily over time. As a result, at 30 and 45 min, the relative potency ratios for these combinations became closer to being equitoxic. Even when 3M2B had its highest relative potency ratio at the 15-min readings, and would be the agent contributing the most to the toxicity of the mixture, the result was not generally consistent with dose-addition. As the relative potency ratios became closer to 1:1 over time, the results showed that actual mixture toxicity was even less consistent with that predicted for dose-addition.

The second feature to note in these data is that EC–AQ values increased over both increasing time of exposure (15, 30, and 45 min) and increasing effect level (25, 50, and 75% effect) for acetonitrile–nonpolar narcotic combinations. The reasons again relate to the time-dependent toxicity differences between the acetonitriles and the nonpolar narcotic and their differences in modes of action.

As noted above, Hodges et al. (2006) also examined mixture toxicity from a perspective of multiple modes of action. While that study differed from this one in that only nonreactive toxicants were evaluated, the results of that study are consistent with those of this study, in that less than dose-additive combined effects were typically observed when chemicals suspected of having different modes of action were tested together. As more studies such as these are conducted, one can expect that the data generated will improve modeling capabilities that will yield better predictions of mixture toxicity.

In summary, the three α -halogenated acetonitriles tested herein: (1) being structurally similar, (2) being S_N2 -reactive with the model nucleophile glutathione, and (3) having time-dependent toxicity of about 100%, showed mixture toxicity consistent with that predicted by the dose-addition model but poorer consistency with that predicted by the independence model. When these same agents were tested with an unreactive nonpolar narcotic agent that lacked an increase in time-dependent toxicity, the combined effects were less toxic than that predicted by either of these combined-effects models. While these results were not unexpected, they serve as a reference for results of the next study to be reported in this series.

In this next study, a second set of S_N2 -reactive agents, the α -halogenated ethyl acetates, will be evaluated using the methodology of the present study but with two notable differences. First, only two of the α -halogenated ethyl acetates have time-dependent toxicity of 100%, while the third has TDT of about 50%. This will allow for examination of mixture toxicity between agents that are reactive by the same mechanism but have different levels of time-dependent toxicity. This should be useful, as it is hypothesized that an agent with mid-range time-dependent toxicity (i.e., between about 20 and 70%) induces toxicity both via a reactive mechanism and through nonpolar narcosis. Secondly, the α -halogenated ethyl acetates are considered to be S_N2 –H-polar agents. The α -halogenated acetonitriles of the present study are S_N2 -reactive, but not H-polar (Roberts et al. 2010). So this difference should provide further insight into mixture toxicity as well, as it will enable comparison of combined effects between S_N2 agents that have the ability to hydrogen-bond strongly with water molecules and those that do not. Since it has been suggested that these *in vivo* reactions may be taking place in different cellular environments (Roberts et al. 2010), it is possible that the resulting mixture toxicity will have distinctive features that could enhance computer modeling of mixture toxicity as it pertains to multiple modes of toxic action.

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Fig. 1. Concentration–response curves for IAN-A alone, IAN-B alone, and the sham mixture after 30 min of exposure, along with predicted curves for dose-addition and independence. The predicted concentration–response curve for dose-addition is almost entirely covered by the actual mixture toxicity curve

Fig. 2. Concentration–response curves for IAN alone, CLAN alone, and their mixture after 45 min of exposure, along with predicted curves for dose-addition and independence. The predicted concentration–response curve for dose-addition is almost entirely covered by the actual mixture toxicity curve

Fig. 3. Concentration–response curves for BRAN alone, 3M2B alone, and their mixture after 30 min of exposure, along with predicted curves for dose-addition and independence

Table 1

Mean values for single chemical reactivity (mM), toxicity (mM), slope, and time-dependent toxicity^a (TDT, %) obtained from Microtox[®] testing

<i>Chemical</i>	<i>RC₅₀</i> ^b	15-min EC ₅₀ (CV) ^c	30-min EC ₅₀ (CV)	45-min EC ₅₀ (CV)	15-min Slope (CV)	30-min Slope (CV)	45-min Slope (CV)	TDT ₁₅₋₃₀	TDT ₁₅₋₄₅	TDT ₃₀₋₄₅
Iodoacetone	0.45	0.018 (2.5)	0.009 (2.5)	0.006 (2.3)	1.53 (1.9)	1.66 (3.5)	1.73 (2.7)	99	99	98
Bromoacetone	0.55	0.024 (3.5)	0.112 (3.5)	0.007 (3.8)	1.51 (2.4)	1.64 (1.8)	1.57 (4.3)	102	106	121
Chloroacetone	13	2.113 (3.1)	1.011 (3.8)	0.630 (6.5)	1.54 (3.2)	1.62 (2.4)	1.63 (3.8)	104	108	110
3-Methyl-2-butanone	Unreactive	0.409 (8.9)	0.434 (6.2)	0.450 (3.7)	1.03 (4.5)	1.01 (4.7)	1.00 (4.7)	-13	-16	-12

^aTDT values were calculated as per Gagan et al. (2007), with TDT subscripts representing the time range for the calculation

^bReactivity as measured with glutathione as the model nucleophile; values are the means of two replicate tests

^cEC₅₀, slope, and TDT values are means for five (IAN, BRAN, 3M2B) or seven (CLAN) separate tests of the agents singly

CV coefficient of variation

Table 2

Relative potency ratio (A:B^a), EC₅₀, and slope dose-additivity quotient (AQ) and EC_x independence quotient (IQ) values for sham^b mixtures tested in Microtox[®]

Sham mixture	Time (min)	A:B	AQ-EC ₂₅	AQ-EC ₅₀	AQ-EC ₇₅	AQ-slope	IQ-EC ₂₅	IQ-EC ₅₀	IQ-EC ₇₅
IAN-IAN ^c	15	1:1.00	0.96	0.98	1.01	0.96	0.79	0.87	1.04
	30	1:0.99	1.00	1.01	1.02	0.99	0.79	0.85	0.98
	45	1:0.99	1.02	1.03	1.03	0.99	0.79	0.85	0.96
BRAN-BRAN	15	1:1.00	1.03	1.02	1.02	1.00	0.82	0.87	0.99
	30	1:0.99	0.99	1.00	1.01	0.98	0.81	0.89	1.04
	45	1:0.99	1.07	1.06	1.04	1.02	0.85	0.89	1.00
CLAN-CLAN #1	15	1:1.00	0.98	1.00	1.03	0.97	0.81	0.90	1.07
	30	1:1.03	0.98	0.99	1.00	0.98	0.80	0.86	1.00
	45	1:1.01	0.99	1.00	1.01	0.98	0.79	0.86	0.99
CLAN-CLAN #2	15	1:0.74	0.98	0.96	0.95	1.03	0.80	0.85	0.95
	30	1:0.77	1.01	0.98	0.95	1.05	0.81	0.84	0.92
	45	1:0.79	1.04	1.01	0.97	1.05	0.83	0.85	0.94
3M2B-3M2B	15	1:1.02	1.01	1.02	1.02	1.00	1.03	1.14	1.38
	30	1:1.05	1.00	0.99	0.98	1.01	1.04	1.15	1.37
	45	1:1.03	1.02	1.05	1.08	0.97	1.05	1.20	1.51

^aRelative potency ratio of the first agent (A) to the second agent (B) in the mixture

^bA sham mixture is prepared from two separate stock solutions of the same chemical and tested as if a true mixture

^cIAN, iodoacetonitrile, BRAN bromoacetonitrile, CLAN chloroacetonitrile, 3M2B 3-methyl-2-butanone

Table 3

Relative potency ratios (A:B^a), EC_x and slope dose-additivity quotient (AQ) and EC_x independence quotient (IQ) values for three halogenated acetoneitrile mixtures tested in Microtox[®]

Mixture	Time (min)	A:B	AQ-EC ₂₅	AQ-EC ₅₀	AQ-EC ₇₅	AQ-slope	IQ-EC ₂₅	IQ-EC ₅₀	IQ-EC ₇₅
IAN-BRAN ^b	15	1:1.22	1.01	1.01	1.00	1.01	0.84	0.90	1.04
	30	1:1.24	1.03	1.03	1.03	1.00	0.83	0.89	1.02
	45	1:1.32	1.04	1.03	1.02	1.02	0.83	0.87	0.98
IAN-CLAN	15	1:0.91	0.97	0.97	0.97	1.00	0.81	0.87	1.00
	30	1:0.96	0.99	0.98	0.97	1.01	0.79	0.84	0.95
	45	1:1.01	1.01	0.99	0.99	1.02	0.79	0.83	0.94
BRAN-CLAN	15	1:0.86	1.09	1.05	1.02	1.05	0.91	0.95	1.06
	30	1:0.87	1.09	1.06	1.04	1.04	0.87	0.91	1.02
	45	1:0.87	1.11	1.08	1.06	1.03	0.88	0.92	1.03

^aRelative potency ratio of the first agent (A) to the second agent (B) in the mixture

^bIAN' iodoacetoneitrile, BRAN' bromoacetoneitrile, CLAN' chloroacetoneitrile, 3M2B 3-methyl-2-butanone

Table 4

Relative potency ratios (A:B^a), EC_x and slope dose-additivity quotient (AQ) and EC_x independence quotient (IQ) values for each of the three halogenated acetoneitriles tested in a binary mixture with a nonpolar narcotic using Microtox[®]

Mixture	Time (min)	A:B	AQ-EC ₂₅	AQ-EC ₅₀	AQ-EC ₇₅	AQ-slope	IQ-EC ₂₅	IQ-EC ₅₀	IQ-EC ₇₅
IAN-3M2B ^b	15	1:3.72	0.95	1.16	1.44	0.80	0.93	1.17	1.23
	30	1:1.73	1.00	1.22	1.49	0.81	0.97	1.10	1.47
	45	1:1.11	1.15	1.30	1.47	0.87	1.07	1.21	1.56
BRAN-3M2B	15	1:3.62	1.04	1.21	1.41	0.85	1.00	1.04	1.20
	30	1:1.65	1.09	1.27	1.52	0.85	1.02	1.13	1.44
	45	1:0.98	1.08	1.30	1.56	0.81	0.98	1.21	1.68
CLAN-3M2B	15	1:1.94	0.87	1.09	1.39	0.78	0.78	0.96	1.32
	30	1:0.92	1.04	1.23	1.46	0.83	0.92	1.16	1.60
	45	1:0.63	1.07	1.30	1.58	0.80	0.96	1.27	1.84

^aRelative potency ratio of the first agent (A) to the second agent (B) in the mixture

^bIAN' iodoacetoneitrile, BRAN' bromoacetoneitrile, CLAN' chloroacetoneitrile, 3M2B 3-methyl-2-butanone