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Halonitromethane Drinking Water Disinfection Byproducts: Chemical Characterization and Mammalian Cell Cytotoxicity and Genotoxicity

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Halonitromethanes are drinking water disinfection byproducts that have recently received a high priority for health effects research from the U.S. Environmental Protection Agency (EPA). Our purpose was to identify and synthesize where necessary the mixed halonitromethanes and to determine the chronic cytotoxicity and the acute genotoxicity of these agents in mammalian cells. The halonitromethanes included bromonitromethane (BNM), dibromonitromethane (DBNM), tribromonitromethane (TBNM), bromochloronitromethane (BCNM), dibromochloronitromethane (DBCNM), bromodichloronitromethane (BDCNM), chloronitromethane (CNM), dichloronitromethane (DCNM), and trichloronitromethane (TCNM). Low- and high-resolution gas chromatography/mass spectrometry (GC/MS) was used to identify the mixed chloro−bromo−nitromethanes in finished drinking waters, and analytical standards that were not commercially available were synthesized (BDCNM, DBCNM, TBNM, CNM, DCNM, BCNM). The rank order of their chronic cytotoxicity (72 h exposure) to Chinese hamster ovary (CHO) cells was DBNM > DBCNM > BNM > TBNM > BDCNM > BCNM > DCNM > CNM > TCNM. The rank order to induce genomic DNA damage in CHO cells was DBNM > BDCNM > TBNM > TCNM > BNM > DBCNM > BCNM > DCNM > CNM. The brominated nitromethanes were more cytotoxic and genotoxic than their chlorinated analogues. This research demonstrated the integration of the procedures for the analytical chemistry and analytical biology when working with limited amounts of sample. The halonitromethanes are potent mammalian cell cytotoxins and genotoxins and may pose a hazard to the public health and the environment.

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Introduction

The disinfection of drinking water was a major public health improvement during the 20th century that reduced the transmission of deadly waterborne diseases (*1*). Drinking water disinfection byproducts (DBPs) are an unintended consequence of chemical disinfection and are formed during water treatment when a disinfectant reacts with organic matter and/or bromide that is naturally present in the water. In 1974 the first DBPs were identified in chlorinated drinking water (*2*). Two years later, a U.S. EPA survey showed that chloroform and other trihalomethanes (THMs) were ubiquitous in chlorinated drinking water (*3*); the same year, the National Cancer Institute published results linking chloroform to cancer in laboratory animals (*4*). The U.S. EPA regulated chloroform and other THMs under the Safe Drinking Water Act (*5*). In almost 30 years following the identification of chloroform, hundreds of DBPs have been identified (*6*). Chloropicrin (trichloronitromethane) was the first of the halonitromethanes to be identified as a DBP and has been measured in several previous studies (*7*−*10*), including EPA's Information Collection Rule monitoring effort (*11*). The first brominated halonitromethane, bromopicrin (tribromonitromethane), was identified approximately 10 years ago and found at ≤2 μg/L levels in a high-bromide water that was ozonated (*12*). Two other brominated analogues, dibromochloronitromethane and dichlorobromonitromethane, had been found previously in reactions of chlorine with model compounds containing nitro groups (*13*) but had not yet been identified as DBPs in drinking water. In 1999, additional brominated halonitromethanes (bromonitromethane and dibromonitromethane) were identified in waters treated with ozone−chlorine and chlorine alone, along with bromopicrin and chloropicrin (*14*, *15*). Most of these were also found in drinking water treated with chlorine or chloramines only (without ozone) but at much lower levels, indicating that ozone may play an important role in their formation. Earlier Hoigné and Bader also observed this effect of ozone on the increased formation of chloropicrin (*7*). In addition, when water with elevated bromide levels (1 ppm) was treated with ozone−chlorine or ozone−chloramines, a shift to more brominated species was observed (*15*). With low bromide levels, chlorinated species dominated (chloro-, dichloro-, and trichloronitromethane); with elevated bromide levels, tremendous increases were observed for brominated species (bromo-, dibromo-, bromochloro-, bromodichloro-, dibromochloro-, and tribromonitromethane) (*15*). Although there is some limited knowledge now regarding the formation of these halonitromethanes in drinking water, little is known about their occurrence and toxicity.

To target DBPs for future health effects studies, the U.S. EPA prioritized a list of ∼500 DBPs according to predicted adverse health effects (*16*). The three bromonitromethanes that had been identified at the time (bromo-, dibromo-, and tribromonitromethane) received the highest ranking and were subsequently included in a nationwide occurrence study. Shortly following this ranking, the remaining five halonitromethanes (chloro-, dichloro-, bromochloro-, bromodichloro-, dibromochloronitromethane) were identified in ozone−chlorine-treated waters (results reported here) and were added as target analytes to the nationwide occurrence study. Quantitative methods developed for the measurement of halonitromethanes in the nationwide occurrence study involved the use of liquid−liquid extraction-GC-electron capture detection (ECD) as the primary method, with solid-phase extraction-GC/MS and purge-and-trap-GC/MS used

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for confirmation of selected species (*17*). Results from the nationwide study supported the finding that ozonation plays an important role in trihalonitromethane formation. Preozonation, in many cases, increased the formation of trihalonitromethanes (following treatment with secondary chloramines) (*18*). Halonitromethanes ranged from 0.1 to 3 μg/L, with tribromo-, bromodichloro-, dibromochloro-, dibromo-, bromochloro-, and bromonitromethane observed (waters high in bromide were targeted in this study) (*17, 18*).

The genotoxicity of three halonitromethanes—chloronitromethane, dichloronitromethane, and trichloronitromethane—have been previously reported (*19*−*22*). These halonitromethanes were more mutagenic in *Salmonella typhimurium* than the corresponding halomethanes, and the bromo-substituted nitromethanes were generally more mutagenic than their chlorinated analogues (*23, 24*). We reported the first preliminary data on the chronic cytotoxicity of bromo-, dibromo-, and tribromonitromethane and the acute genotoxicity of bromo- and dibromonitromethane in mammalian cells (*25*).

The objectives of this research were to (i) identify the mixed chloro−bromo−nitromethanes in drinking water, (ii) synthesize halonitromethane analytical standards that were not commercially available, (iii) report the analytical chemistry for the analysis and identification of this class of DBPs, (iv) analyze and rank order the cytotoxic potency of these agents in mammalian cells, and, (v) determine the genotoxic potency of the halonitromethanes in mammalian cells.

Experimental Section

Drinking Water Samples. Drinking water samples treated with ozone−chlorine, ozone−chloramines, chloramine, and chlorine were collected from a pilot plant in Jefferson Parish, LA, which used Mississippi River water as the raw water source (2.6−3.0 mg/L total organic carbon; 0.054 mg/L bromide). Samples (75 L for analytical identification work) were acidified to pH 2 and concentrated immediately after collection using XAD-8 over XAD-2 resins, eluted with ethyl acetate and concentrated to 1 mL using rotary evaporation followed by evaporation under nitrogen (*14*).

GC/MS Analysis. GC/MS analyses with electron ionization (EI) were performed on a VG 70-SEQ high-resolution mass spectrometer equipped with a HP 5890A gas chromatograph. The high-resolution mass spectrometer was operated at an accelerating voltage of 8 kV and at resolutions of 1 000 and 10 000 for low- and high-resolution experiments, respectively. Perfluorokerosene (pfk) was used as the mass calibrant. Injections of 1−2 μL of the extracts (or standard solutions in ethyl acetate) were introduced via a split/splitless injector onto a J&W Scientific DB-5 column (30 m, 0.25 mm i.d., 0.25 μm film thickness). The GC temperature program consisted of an initial temperature of 35 °C for 4 min, followed by a rate increase of 9 °C/min to 285 °C, which was held for 30 min. An injection port temperature of 170 °C and a GC/MS transfer line temperature of 200 °C were used to analyze trihalonitromethanes (to prevent thermal decomposition) (*26*).

Chemical Standards. Bromonitromethane (90% pure) and trichloronitromethane (98% pure) were purchased from Aldrich Chemical Co. (St. Louis, MO). Dibromonitromethane (98% pure) was provided by Majestic Research (Athens, GA) (*15*). Bromodichloronitromethane and dibromochloronitromethane were prepared by the addition of nitromethane to commercial bleach, to which a solution of bromine in 6 M NaOH had been previously added (*27*). The products were separated by fractional distillation under reduced pressure. Tribromonitromethane was prepared by bromination of nitromethane in 1 M NaOH (*28*). The reaction product was isolated by steam distillation to give colorless material, bp 85−86 °C at 20 mm (lit. 85 °C at 17 mm) (*29*). Chloronitromethane was prepared from the ammonium salt of methyl nitroacetate, as reported for the ethyl ester (*30*), as follows: sulfuryl chloride (7.4 g, 0.55 mol) in ether (10 mL) was added to a stirred suspension of the powdered salt (6.8 g, 0.05 mol) in ether (50 mL) at room temperature. After 15 min, the ammonium chloride was filtered and the filtrate washed with water, dried, and concentrated to give crude methyl chloronitroacetate (7 g). The crude product was refluxed with 10% hydrochloric acid (HCL) (14 mL) with stirring for 1.5 h. The product was cooled, extracted with ether, washed with water, and dried. The ether was removed by distillation through a 10 cm Vigreux column, the column removed, and the product distilled to give chloronitromethane (1.6 g), bp 122−123 °C (lit. bp 122−123 °C) (*31*). Dichloronitromethane was prepared from the ammonium salt of methyl nitroacetate as follows: chlorine (3.6 g, 0.05 mol) was bubbled into a stirred solution of the salt (6.8 g, 0.05 mol) in water (60 mL) over 10 min at room temperature. After another 10 min, the insoluble product oil was extracted with ether, washed with water, and dried. Anhydrous ammonia was bubbled into the ether solution of the product, and the ammonium salts of unreacted methyl nitroacetate and methyl chloronitroacetate were filtered. The salts were dissolved in water and chlorinated again as described above. The ether extract of the second chlorination was treated again with anhydrous ammonia to remove unreacted nitroacetates. This filtrate was combined with the filtrate from the first chlorination, and the combined filtrates were washed with water, dried, and concentrated to give crude methyl dichloronitroacetate (6.3 g). The crude product was refluxed with 20% HCL (13 mL) with stirring for 3.5 h. The product was processed as described above for chloronitromethane to give dichloronitromethane (2.2 g), bp 108−110 °C (lit. bp 110−112 °C, 108−110 °C) (*32*). Bromochloronitromethane was prepared as follows: anhydrous ammonia was bubbled into an ether solution of crude methyl chloronitroacetate (6.1 g, 0.04 mol) prepared as described above. The resultant ammonium salt (6.5 g) was dissolved in water (65 mL) and treated with bromine (6.4 g, 0.04 mol) with stirring at room temperature. After 10 min, the insoluble product oil was extracted with ether, dried, and concentrated to give crude methyl bromochloronitroacetate (7 g). The product was refluxed with 20% sulfuric acid (15 mL) with stirring for 3 h. After workup as described above for chloronitromethane and removal of most of the ether by distillation through a 10 cm Vigreux column, the residue (5 g) was fractionated on silica gel (150 g) and the product eluted with 9:1 pentane:ether. The solvent was distilled through the column, the column removed, and the product distilled to give bromochloronitromethane (0.7 g), bp 94−97 °C at 160 mm. Purities of these synthesized halonitromethanes by GC using flame ionization detection were as follows: chloronitromethane (97%), dichloronitromethane (99%), bromodichloronitromethane (92%), dibromochloronitromethane (97%), tribromonitromethane (96%), and bromochloronitromethane (90%).

Cell Culture Medium and Biologicals. General laboratory reagents were purchased from Fisher Scientific Co. (Itasca, IL) and Sigma Chemical Co. (St Louis, MO). Media supplies and fetal bovine serum (FBS) were purchased from Hyclone Laboratories (Logan, UT). Clone 11−4−8 of Chinese hamster ovary (CHO) cell line AS52 were maintained in Ham's F12 medium containing 5% FBS, 1% antibiotics (100 U/mL sodium penicillin G, 100 μg/mL streptomycin sulfate, 0.25 μg mL amphotericin B, 0.85% saline), and 1% glutamine at 37 °C in a humidified atmosphere of 5% CO_2 (*33*). For long-term storage, the cells were frozen in FBS:dimethyl sulfoxide (9:1, v/v) and kept at −80 °C.

Microplate Cytotoxicity Assay. Chronic cytotoxicity to mammalian cells was measured using a modification of an assay we developed for the analysis of DBPs (*25*, *34*). Flat-

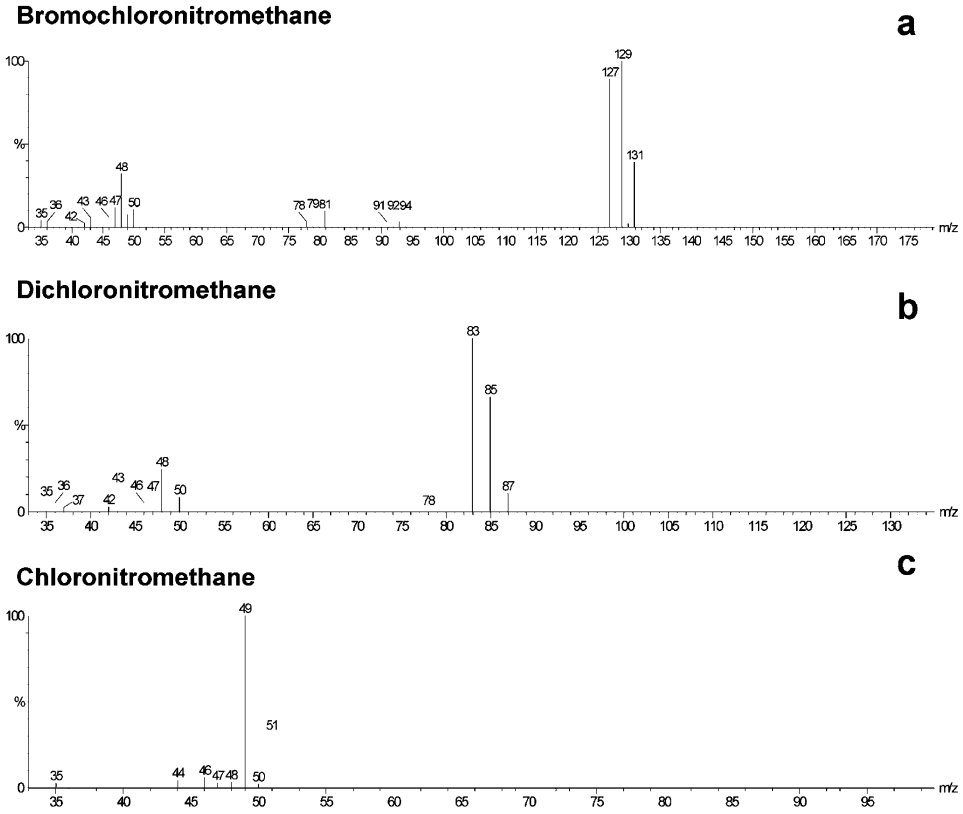

FIGURE 1. Electron ionization mass spectra for bromochloronitromethane (a), dichloronitromethane (b), and chloronitromethane (c).

bottom, tissue culture 96-well microplates were employed; eight replicate wells were prepared for each concentration of a specific DBP. Eight wells were reserved for the blank control consisting of 200 μL of F12 medium + 5% FBS. The negative control consisted of eight wells containing 100 μL of a titered CHO cell suspension (3×10^4 cells/mL) plus 100 μL F12 + FBS. The wells for the remaining columns contained 3000 CHO cells, F12 + FBS, and a known concentration of a DBP for a total of 200 μL. To prevent crossover contamination between wells due to volatilization of the test agent, a sheet of sterile AlumnaSeal (RPI Corporation, Mt. Prospect, IL) was pressed over the wells before the microplate was covered. The plate was placed on a rocking platform for 10 min to uniformly distribute the cells, and the microplate was placed in a tissue culture incubator for 72 h. Each well was gently aspirated, fixed in 100% methanol for 10 min, and stained for 30 min with a 1% crystal violet solution in 50% methanol. The plate was gently washed, and 50 μL of DMSO was added to each well for 30 min. The plate was analyzed in a BioRad microplate reader at 595 nm. The data were automatically recorded and transferred to an Excel spreadsheet in a microcomputer connected to the microplate reader.

Single-Cell Gel Electrophoresis (SCGE) Assay. The day before treatment, 2×10^4 CHO cells were added to each microplate well in 200 μL of F12 + 5% FBS and incubated. The next day the cells were washed with Hank's balanced salt solution (HBSS) and treated with the halonitromethane in F12 medium without FBS in a total volume of 25 μL for 4 h at 37 °C, 5% CO_2. The wells were covered with sterile AlumnaSeal. After incubation, the cells were washed 2× with HBSS and harvested with 50 μL of 0.005% trypsin + 53 μM EDTA. The trypsin was inactivated with 70 μL of F12 + FBS. A 10 μL aliquot was removed to measure acute cytotoxicity using 10 μL of 0.05% trypan blue vital dye in phosphate-buffered saline (PBS). SCGE data were not used if the acute cytotoxicity exceeded 30%. The remaining cell suspension from each well was embedded in a layer of low-melting point agarose prepared with PBS upon clear microscope slides that were previously coated with a layer of 1% normal melting point agarose prepared with deionized water and dried overnight. The detailed methods for preparing and electrophoresing the SCGE slides were published previously (*34*). The cellular membranes were removed by an overnight immersion in lysing solution at 4 °C. The slides were placed in an alkaline buffer (pH 13.5) in an electrophoresis tank, and the DNA was denatured for 20 min. The slides were electrophoresed at 25 V, 300 mA (0.72 V/cm) for 40 min at 4 °C. The slides were removed, neutralized with Tris buffer, pH 7.5, rinsed in cold water, dehydrated in cold methanol, dried at 50 °C, and stored at room temperature in a covered slide box. For analysis, the slides were hydrated in cold water for 20 min and stained with 65 μL of 2 μg/mL ethidium bromide for 3 min. The slides were rinsed in cold water and analyzed with a Zeiss fluorescence microscope with an excitation filter of BP 546/10 nm and a barrier filter of 590 nm. For each experiment two slides were prepared per treatment group. The slides were coded, and 25 randomly chosen nuclei were analyzed in each slide using a charge-coupled device camera. A computerized image analysis system (Komet version 3.1, Kinetic Imaging Ltd., Liverpool, U.K.) was employed to determine the tail moment (integrated value of migrated DNA density multiplied by the migration distance) of the nuclei as an index of DNA damage. The digitalized data were automatically transferred to a computer-based spreadsheet for subsequent statistical analysis.

Safety and Data Handling. Manipulations of toxic and mutagenic chemicals were conducted in certified biological/chemical safety hoods. In general, for the chronic cytotoxicity assays, the experiments were repeated at least twice with a minimum of eight independent replicates for each chemical concentration per experiment, and for the SCGE assay three experiments were conducted with six slides analyzed per treatment group. The median tail moment value for each slide was determined, and the data from all of the slides

TABLE 1. Important Mass Spectral Ions for Halonitromethanes

compd	monoisotopic mol wt	important MS ions (*m/z*, relative abundance)
bromonitromethane	139	30(41), 43(4), 44(13), 46(10), 79(10), 81(10),93(100), 95(95)
chloronitromethane	95	43(9), 44(10), 46(12), 49(100), 51(42)
bromochloronitromethane	173	43(23), 46(9), 47(18), 48(38), 49(11), 50(15), 79(13), 81(13), 92(12), 94(12), 127(87), 129(100), 131(37)
dibromonitromethane	217	43(18), 46(10), 79(30), 81(30), 92(45), 94(45), 122(4), 124(4), 158(2), 160(4), 162(2), 171(67), 173(100), 175(63)
dichloronitromethane	129	43(22), 46(8), 47(15), 48(33), 49(7), 50(12), 83(100), 85(71), 87(13)
bromodichloronitromethane	207	36(12), 46(20), 47(41), 49(19), 61(20), 63(12), 77(8), 79(17), 81(15), 82(36), 84(25), 91(8), 93(8), 126(17), 128(23), 130(9), 161(70), 163(100), 165(55), 167(13)
dibromochloronitromethane	251	36(7), 46(11), 47(27), 49(12), 61(6), 63(4), 79(20), 81(20), 91(18), 93(18), 105(4), 107(4), 126(30), 128(34), 130(11), 170(6), 172(10), 174(7), 205(55), 207(100), 209(77), 211(24)
tribromonitromethane (bromopicrin)	295	36(13), 38(5), 46(6), 79(22), 81(22), 91(35), 93(35), 105(6), 107(7), 121(10), 123(10), 170(24), 172(42), 174(23), 249(44), 251(100), 253(98), 255(41)
trichloronitromethane (chloropicrin)	163	46(1), 47(28), 49(9), 61(10), 63(8), 65(2), 82(33), 84(21), 86(4), 117(100), 119(96), 121(30)

TABLE 2. CHO Cell Chronic Cytotoxicity Induced by the Halonitromethanes

compd	ANOVA test statistic	$\%C^1/_2$ value (M)[a]	r^2 [b]	rank order
bromonitromethane	$F_{11,68} = 23.19$ $P \leq 0.001$	7.06×10^{-6}	0.85	3
dibromonitromethane	$F_{8,78} = 56.60$ $P \leq 0.001$	6.09×10^{-6}	0.99	1
tribromonitromethane	$F_{9,17} = 19.72$ $P \leq 0.001$	8.57×10^{-6}	0.99	4
chloronitromethane	$F_{16,95} = 41.53$ $P \leq 0.001$	5.29×10^{-4}	0.87	8
dichloronitromethane	$F_{16,47} = 35.24$ $P \leq 0.001$	3.73×10^{-4}	0.91	7
trichloronitromethane	$F_{19,91} = 16.54$ $P \leq 0.001$	5.36×10^{-4}	0.94	9
bromochloronitromethane	$F_{18,47} = 15.24$ $P \leq 0.001$	4.05×10^{-5}	0.82	6
bromodichloronitromethane	$F_{31,96} = 10.25$ $P \leq 0.001$	1.32×10^{-5}	0.81	5
dibromochloronitromethane	$F_{28,78} = 29.21$ $P \leq 0.001$	6.88×10^{-6}	0.90	2

[a] The $\%C^1/_2$ value is the chemical concentration that induced a 50% reduction of the cell density as compared to the negative control. [b] r^2 refers to the fit of the regression analysis upon which the $\%C^1/_2$ value was calculated.

representing each halonitromethane concentration were averaged. Averaged median values express a normal distribution according to the central limit theorem and were used with a one-way analysis of variance test (*35*). If a significant F value of $P \leq 0.05$ was obtained, a Dunnett's multiple comparison versus the control group analysis was conducted. In most cases, the power of the test statistic (β) was ≥ 0.8 at $\alpha = 0.05$. To analyze the strength of association between pairs of variables, we used the Pearson Product Moment Correlation test.

Results and Discussion

The chemistry and toxicity of DBPs have been investigated for over a quarter century. However, many important classes of DBPs, such as the halonitromethanes, have not been adequately studied for their cytotoxicity and genotoxicity. We integrated the analytical chemistry with the analytical biology in the first comprehensive evaluation of the toxicity and genotoxicity of halonitromethane DBPs using an in vitro mammalian cell system.

Analytical Chemistry. The first identification of chloro-, dichloro-, bromochloro-, bromodichloro-, and dibromochloronitromethane in drinking water is reported here. They were primarily found in waters treated with ozone−chlorine. In general, the identification of the halonitromethanes was not trivial. Only bromonitromethane and trichloronitromethane were found in the NIST or Wiley spectral library database. The spectra generated here were recently submitted to NIST for inclusion in an upcoming database release. Another complicating factor was that none of the halonitromethanes show molecular ions in their mass spectra; the molecular weight information and the overall composition of the structures are missing. Each of these compounds loses a $NO_2^{\bullet}$, leaving the remaining fragment as the highest mass ion (e.g., tribromonitromethane loses $NO_2^{\bullet}$ to form a CBr_3^+ as the highest mass ion). There is only the slightest hint of the presence of the NO_2 in their structures, with small (usually $\leq$10% relative abundance) m/z 46 ions in their mass spectra. The presence of another ion at m/z 43 also helped to identify these compounds. This ion was present in the mono- and dihalonitromethanes and is typically a C_3H_7 group for most compounds. However, the m/z 43 ion in this case was due to the uncommon $CHNO^+$ ion, as determined by high-resolution EI-MS (observed exact mass = 43.006 Da, theoretical mass for CHNO = 43.006 Da; theoretical mass for C_3H_7 = 43.055 Da). These data suggested the presence of an NO_2 group in the structure of these compounds. Mass spectra of three halonitromethanes (bromochloro-, dichloro-, and chloronitromethane) are shown in Figure 1; mass spectra of the other halonitromethanes can be found in a recent

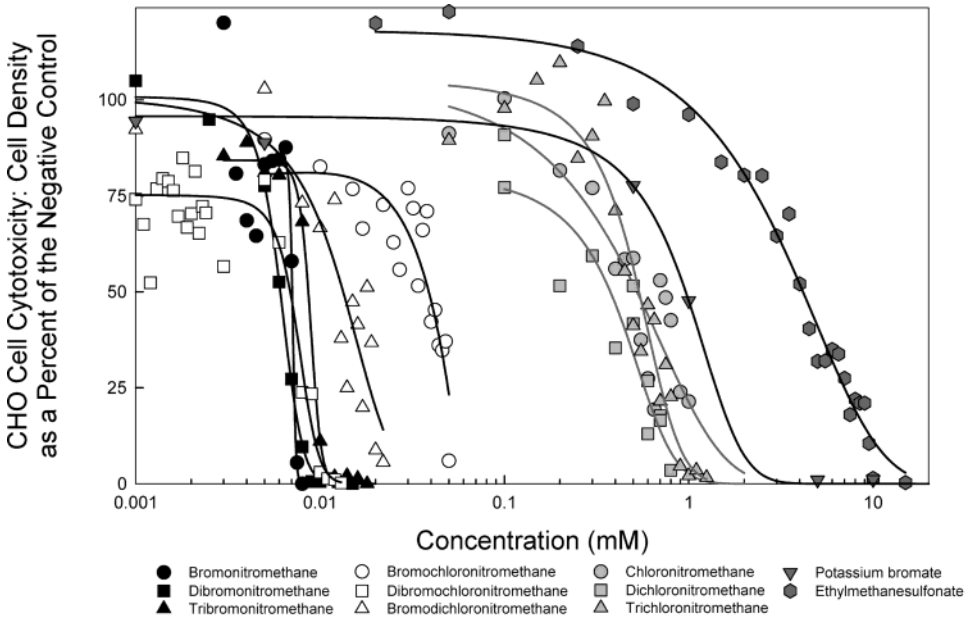

FIGURE 2. Log-linear plot of the chronic CHO cell cytotoxicity induced by the halonitromethanes and the positive controls EMS and potassium bromate. The statistical analysis of these data are presented in Table 2.

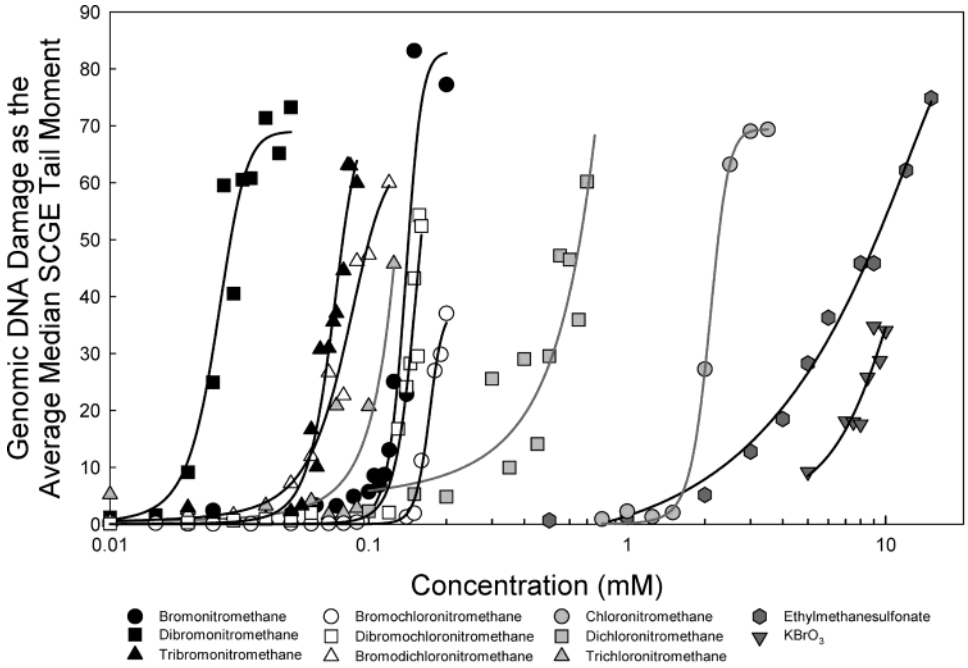

FIGURE 3. Log-linear plot of the SCGE tail moment data showing genomic DNA damage induced by the halonitromethanes and the positive controls EMS and potassium bromate. The statistical analysis of these data are presented in Table 3.

publication that details their thermal decomposition behavior when analyzed by GC or GC/MS (*26*). Table 1 lists important mass spectral ions for the halonitromethanes. After we tentatively identified nine halonitromethanes, we purchased standards for the commercially available bromonitromethane and trichloronitromethane and synthesized the others. GC/MS retention times and mass spectra of the pure standards matched those in our drinking water samples, confirming their identities.

The trihalonitromethanes required special conditions for detection. They are thermally unstable and decompose under commonly used GC injection port temperatures (200−250 °C) (*12*), forming radical ions and radical reaction products (*26*). The trihalonitromethanes also thermally decompose in the GC/MS transfer line at commonly used transfer line temperatures (≥250 °C), producing mass spectra that are mixtures of the undecomposed parent compound and decomposition products. For example, at a GC/MS transfer line temperature of 290 °C, tribromonitromethane exhibited a mixed mass spectrum composed of tribromonitromethane and bromoform. The trihalonitromethanes also show unusual $[\text{fragment} + 1]^+$ ions in their mass spectra, which can complicate their identification. We recommend that low GC injection port temperatures (≤170 °C) and low transfer line temperatures (≤225 °C) be used for their analysis (*26*). This behavior was observed only for the trihalonitromethanes and not for the mono- or dihalonitromethanes.

Brominated nitromethanes were also found in the recent U.S. Nationwide DBP Occurrence Study. Individual halonitromethanes ranged from 0.1 to 3 μg/L, with tribromo-,

TABLE 3. CHO Cell Genotoxicity Induced by the Halonitromethanes

compd	ANOVA test statistic	SCGE genotoxic potency (M)[a]	r^2 [b]	rank order
bromonitromethane	$F_{14,42} = 85.96$ $P \leq 0.001$	1.36×10^{-4}	0.88	5
dibromonitromethane	$F_{11,54} = 39.43$ $P \leq 0.001$	2.62×10^{-5}	0.94	1
tribromonitromethane	$F_{14,43} = 9.02$ $P \leq 0.001$	6.99×10^{-5}	0.95	3
chloronitromethane	$F_{9,27} = 60.58$ $P \leq 0.001$	2.15×10^{-3}	0.99	9
dichloronitromethane	$F_{8,36} = 61.21$ $P \leq 0.001$	4.21×10^{-4}	0.97	8
trichloronitromethane	$F_{10,20} = 25.76$ $P \leq 0.001$	9.34×10^{-5}	0.86	4
bromochloronitromethane	$F_{7,22} = 30.42$ $P \leq 0.001$	1.65×10^{-4}	0.97	7
bromodichloronitromethane	$F_{6,14} = 13.3$ $P \leq 0.001$	6.32×10^{-5}	0.98	2
dibromochloronitromethane	$F_{8,29} = 9.02$ $P \leq 0.001$	1.43×10^{-4}	0.95	6

[a] The genotoxic potency is the chemical concentration at the midpoint of the SCGE concentration−response curve. [b] r^2 refers to the fit of the regression analysis upon which the SCGE genotoxic potency value was calculated.

bromodichloro-, dibromochloro-, dibromo-, bromochloro-, and bromonitromethane observed (waters high in bromide were targeted in this study) (*17*, *18*). It is likely that bromo- and mixed chloro−bromo−nitromethanes are present in many drinking waters treated with ozone−chlorine, ozone−chloramines, chlorine, or chloramines and that they have been largely undetected due to the difficulty in identifying them, the lack of analytical standards, and the special instrumental conditions required for their identification.

Analytical Biology. For the first time, the chronic cell cytotoxicity and acute genotoxicity in a mammalian system of a complete set of bromo- and chloro-substituted halonitromethanes were determined. The microplate cytotoxicity assay measured the reduction in cell density as a function of the halonitromethane concentration over a 72 h period. A reduction in cell density could be due to a disruption in the cell cycle, growth retardation, or cell toxicity. We refer to the halonitromethane concentration that causes a 50% reduction of the cell density as compared to the negative control as the $\%C^1/_2$ value rather than the LC_{50}.

The data from repeated cytotoxicity experiments were averaged and plotted (Figure 2), and regression analysis was used to calculate the $\%C^1/_2$ value for each halonitromethane (Table 2). There was an approximately 100-fold range in the $\%C^1/_2$ values from a value of 6.09 μM for dibromonitromethane to 536 μM for trichloronitromethane (Table 2). All of the halonitromethanes were more cytotoxic than the positive controls ethylmethanesulfonate (EMS) ($\%C^1/_2$ = 4.19 mM) and potassium bromate ($\%C^1/_2$ = 964 μM) (Figure 2). The rank order in declining toxicity demonstrated that the brominated nitromethanes were more cytotoxic than their chlorinated analogues. It appeared that the multibrominated halonitromethanes were the most toxic. For both the bromo- and chloronitromethanes, the rank order in declining cytotoxicity was the di-, mono-, and trihalogenated forms (Table 2). In previous work we found that the brominated haloacetic acids were more cytotoxic than their chlorinated analogues (*34*). In all cases, the halonitromethanes were more cytotoxic than the corresponding haloacetic acids. Bromonitromethane and chloronitromethane were 1.25× and 1.8× more cytotoxic than bromoacetic acid and chloroacetic acid, respectively. Dichloronitromethane and trichloronitromethane were more cytotoxic than dichloroacetic acid and trichloroacetic acid (29.5× and 32.6×, respectively). The results were the most striking for dibromonitromethane and tribromonitromethane, 83× and 116× more cytotoxic than dibromoacetic acid and tribromoacetic acid.

We compared the chronic cytotoxicity of the halonitromethanes in CHO cells with qualitative cytotoxicity reported for *S. typhimurium* (*23*, *24*) and found no significant correlation ($r = 0.60$; $P = 0.09$). This is similar to data published with the bromo- and chloro-substituted haloacetic acids in that one cannot use *Salmonella* cytotoxicity data to predict mammalian cell cytotoxicity (*34*, *36*).

There are very few studies on the genotoxicity of halonitromethanes in mammals or mammalian cells. SCGE is a very sensitive assay that can quantitatively determine genomic DNA damage (*37*). The halonitromethanes were potent mammalian cell genotoxins (Figure 3). The SCGE genotoxic potency was calculated for each halonitromethane as the midpoint in the concentration−response curves (Figure 3, Table 3). All nine of the halonitromethanes exceeded the genotoxicity of the positive control mutagen EMS in a range from 260× for dibromonitromethane to 3× for chloronitromethane. The halonitromethanes were more potent DNA-damaging agents than potassium bromate. Potassium bromate was reported as an oxidative stress-inducing genotoxin (*38*) and animal carcinogen (*39*). The genotoxic potency of potassium bromate was 7.2 mM (*34*); the halonitromethanes were between 275× and 3× more genotoxic (dibromonitromethane and chloronitromethane, respectively). From a structure−function perspective, the brominated nitromethanes and the mixed bromo−chloro−nitromethanes were more genotoxic than the chlorinated nitromethanes. The mono-, di-, and tribrominated nitromethanes were 16×, 16×, and 1.3× more genotoxic than their chlorinated analogues. A comparison of the mutagenicity of these halonitromethanes in *S. typhimurium* (*23*, *24*) and genomic DNA damage in CHO cells exhibited no significant correlation ($r = -0.003$; $P = 0.99$). These data indicate that mutagenicity in *S. typhimurium* cannot predict genotoxicity in mammalian cells, and this conclusion agrees with that reported for the haloacetic acids (*34*). The halonitromethane DBPs, especially the bromo-substituted forms, are potent mammalian cell genotoxins and may pose a hazard to the public health and the environment.

Concentrations observed in the nationwide occurrence study (*17*, *18*) can help to put the relative toxicities of the halonitromethanes in perspective. For example, the maximum occurrence of the monobrominated halonitromethane species (bromonitromethane) was 0.3 μg/L, whereas the maximum occurrence of the corresponding haloacetic acid (bromoacetic acid) was 1.6 μg/L. For this maximum occurrence, bromoacetic acid was 5.3× higher in concentration,

but bromonitromethane was 1.26× more cytotoxic and 8× more genotoxic. Similarly, the maximum occurrence of the dichloronitromethane was approximately 1 μg/L, as compared to 27 μg/L for dichloroacetic acid. Thus, dichloroacetic acid was at maximum levels 27× that of the dichloronitromethane, but the dichloronitromethane was 30.8× more cytotoxic. Dichloronitromethane was genotoxic in CHO cells, while dichloroacetic acid was refractory. The maximum occurrence of dibromonitromethane was 0.4 μg/L, as compared to 18 μg/L for dibromoacetic acid (45×), but dibromonitromethane was 82.6× more cytotoxic and 67.2× more genotoxic than dibromoacetic acid (*34*). These limited occurrence data suggest that when both relative concentrations and toxicities are considered, the halonitromethanes are as important as the regulated haloacetic acids.

As mentioned earlier, preozonation (followed by chlorination or chloramination) appears to increase the formation of some halonitromethanes, which had been observed in previous studies of chloropicrin (*7*). Mechanistic studies are currently underway to determine how these halonitromethanes are formed in order to ultimately minimize their formation in drinking water.

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