Environmental Health Perspectives
Vol. 69, pp. 67-71, 1986

Haloacetonitriles: Metabolism, Genotoxicity, and Tumor-Initiating Activity

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Haloacetonitriles (HAN) are drinking water contaminants produced during chlorine disinfection. This paper evaluates metabolism, genotoxicity, and tumor-initiating activity of these chemicals. The alkylating potential of the HAN to react with the electrophile-trapping agent, 4-(*p*-nitrobenzyl)pyridine, followed the order dibromoacetonitrile (DBAN) > bromochloroacetonitrile (BCAN) > chloroacetonitrile (CAN) > dichloroacetonitrile (DCAN) > trichloroacetonitrile (TCAN). When administered orally to rats, the HAN were metabolized to cyanide and excreted in the urine as thiocyanate. The extent of thiocyanate excretion was CAN > BCAN > DCAN > DBAN >> TCAN. Haloacetonitriles inhibited *in vitro* microsomal dimethylnitrosamine demethylase (DMN-DM) activity. The most potent inhibitors were DBAN and BCAN, with K_i = 3–4 × 10^{-5} M; the next potent were DCAN and TCAN, with K_i = 2 × 10^{-4} M; and the least potent inhibitor was CAN, with K_i = 9 × 10^{-2} M. When administered orally, TCAN, but not DBAN, inhibited hepatic DMN-DM activity.

The HAN produced DNA strand breaks in cultured human lymphoblastic (CCRF-CEM) cells. TCAN was the most potent DNA strand breaker, and BCAN > DBAN > DCAN > CAN, which was only marginally active. DCAN reacted with polyadenylic acid and DNA to form adducts in a cell-free system; however, the oral administration of DBAN or DCAN to rats did not result in detectable adduct formation in liver DNA. None of the HAN initiated γ-glutamyltranspeptidase (GGT) foci when assayed for tumor-initiating activity in rat liver foci bioassay. In summary, the HAN were demonstrated to possess alkylating activity and genotoxicity *in vitro* and appeared after oral administration to possess biological activity as indicated by the inhibition of DMN-DM by TCAN but appeared to lack genotoxic and tumor-initiating activity in rat liver. It is proposed that if the HAN found in drinking water pose a carcinogenic hazard it would be limited to the gastrointestinal tract.

Introduction

Chlorine is routinely used as a municipal drinking water disinfectant. However, chlorination of drinking water produces a variety of chlorinated organic compounds (*1*), including trihalomethanes and halogenated acetonitriles (HAN). Dichloroacetonitrile (DCAN), the most prevalent of the haloacetonitriles, has been routinely detected in drinking water at 0.3–8.1 ppb, which is about 10% of the molar concentration for trihalomethanes (*2*). DCAN has been reported (*3*) to be mutagenic in *Salmonella typhimurium*, so that exposure of humans to haloacetonitriles via drinking water might present a genotoxic and carcinogenic hazard. Thus the metabolism, genotoxicity, and tumor-initiating activity of HAN were evaluated.

Results

Alkylation Potential

The HAN have been demonstrated to react with the electrophile-trapping agent 4-(*p*-nitrobenzyl)pyridine (NBP) to form a colored product (*4*). Linear dose-response curves were obtained when absorbance at 570 nm of the colored product was plotted against five different concentrations of HAN. The extent of reaction was calculated as the slope of absorbance at 570 nm vs. the μ M test chemical curve after a linear-regression fit of the data. The results were standardized to chloroacetonitrile (CAN) equal to one and are presented in Table 1. CAN was the most active among the chlorinated HAN, and each additional chlorine substitution reduced reactivity by a factor of approximately ten. Substitution of chlorine by bromine increased reactivity, and DBAN was 100 times more reactive than DCAN. These results were similar to those obtained and cited by Epstein et al. (*5*) with alkyl halides: RCH_2CH_2X > RCH_2CHX_2 > RCH_2CX_3 and iodide > bromide > chloride.

Urinary Excretion of HAN as Thiocyanate

The thiocyanate content of 24-hr urine samples was determined after oral administration of 0.75 mmole/kg of HAN (*6*). The percentage of the dose excreted in the urine as thiocyanate was standardized to CAN equal to

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Table 1. Summary of the relative biological and chemical activities of some haloacetonitriles.

HAN	Alkylation potential[a]	Urinary excetion[b]	Inhibition of DMN-DM[c]	DNA strand breaks[d]
CAN	1.00	1.0	1	1.0
DCAN	0.068	0.7	450	2.1
BCAN	2.2	0.9	2300	6.3
DBAN	6.2	0.2	3000	3.4
TCAN	0.010	0.2	450	37.0

[a] Alkylation potential as determined by binding to 4-(*p*-nitrobenzyl)pyridine (*4*).
[b] Urinary excretion as thiocyanate in 24 hr (*6*).
[c] Inhibition of microsomal DMN-DM (*6*).
[d] DNA strand breaks induced in CCRF-CEM cells of human lymphoblastic line of T-cell origin (*4*).

one (Table 1). CAN and bromochloroacetonitrile (BCAN) were excreted to a greater extent than DCAN and dibromoacetonitrile (DBAN), which were excreted to a greater extent than trichloroacetonitrile (TCAN). Figure 1 depicts a proposed pathway for the metabolism of HAN to cyanide, which is further metabolized by rhodanese to thiocyanate. Hydroxylacetonitriles were proposed to form either by direct displacement of halide ion by an hydroxyl group or by oxidation of a hydrogen atom through the action of mixed-function oxidase. The carbonyl group is formed from the hydroxylacetonitrile by the elimination of cyanide ion or by the release of halide ion, forming cyanoformaldehyde or cyanoformyl halide. Cyanide ion could then be formed by the release of carbon monoxide from cyanoformaldehyde or by release of carbon dioxide from cyanoformic acid, which is formed by the hydrolysis of cyanoformyl halide. A similar metabolic pathway for the release of cyanide from nitriles has been proposed by Silver et al. (*7*).

Inhibition of Dimethylnitrosamine Demethylase

The HAN inhibited rat microsomal dimethylnitrosamine-demethylase (DMN-DM) activity *in vitro* (*6*). The dose-response relationship of inhibition by HAN of DMN-DM activity is presented in Figure 2. DBAN and

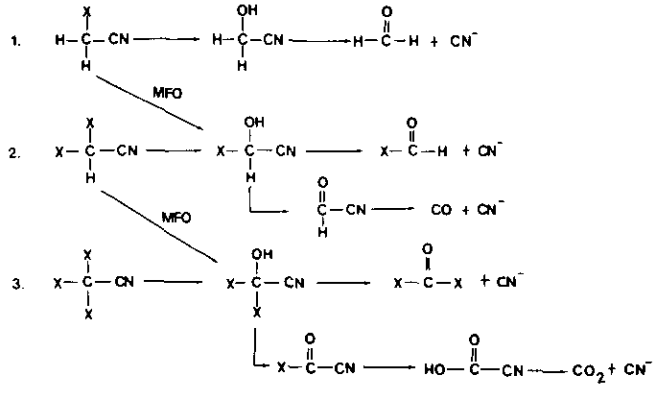

FIGURE 1. Proposed metabolic pathways for the formation of cyanide from the HAN. From Pereira et al. (*6*)

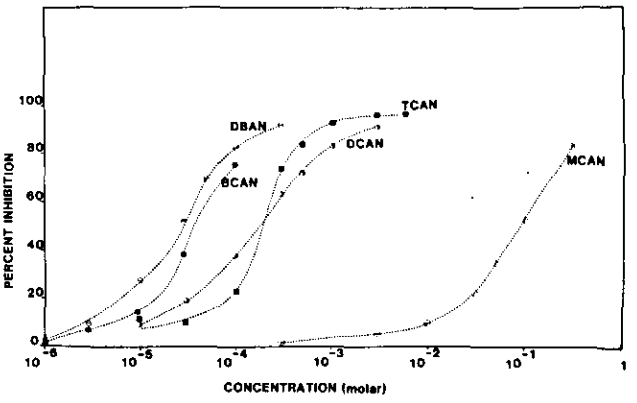

FIGURE 2. Dose-response relationships of the inhibition of DMN-DM by the HAN. From Pereira et al. (*6*).

BCAN were the most potent inhibitors, with K_i = 3–4 × 10^{-5} M; DCAN and TCAN were the next most potent inhibitors, with $K_i = 2 \times 10^{-4}$ M; and CAN was the least potent inhibitor with $K_i = 9 \times 10^{-2}$ M. Table 1 contains the relative potencies of the HAN inhibition of DMN-DM standardized to CAN = 1.00. The effect of *in vivo* administration of DBAN and TCAN on DMN-DM activity in rat liver is presented in Table 2 (*6*). TCAN, but not DBAN, inhibited DMN-DM activity at both 3 hr and 18 hr.

DNA Strand Breaks in Human Lymphoblastic Cells

The HAN were incubated with CCRF-CEM cells, a human lymphoblastic line of T-cell origin, and the induction of DNA strand breaks was assayed by the alkaline unwinding procedure (*4*). All five HANs were able to induce DNA strand breaks. The extent of DNA strand breaks induced by the five HAN after 1 hr of exposure was standardized to CAN = 1.00 and is presented in Table 1. The HAN exhibited a range of potency, with TCAN the most potent, causing twice as many breaks as the genotoxic methylating agents methylmethanesulfonate and methylnitrosourea (*4*). The two bromine-containing HAN were cytotoxic to the CCRE-CEM cell, producing a 13 to 60% rate of cell

Table 2. Effect of DBAN and TCAN on rat liver DMN-DM activity.[a]

HAN	Demethylase activity[b]	
	3 hr	18 hr
DBAN	90.2 ± 3.9	89.3 ± 5.4*
TCAN	48.1 ± 2.7†	47.3 ± 3.1†
Tricaprylin	77.9 ± 1.3	71.3 ± 4.5

[a] Male Sprague-Dawley rats were administered by gavage 0.75 mmole/kg of DBAN or TCAN dissolved in tricaprylin, and DMN-DM activity was determined in isolated microsomes. Adapted from Pereira et al. (*6*).
[b] Results are means of 6 rats for the Han and 12 rats for the tricaprylin.
*Different from control by Student's *t*-test, $p < 0.01$.
†Different from control by Student's *t*-test, $p < 0.001$.

killing at 50 μm. The cytotoxicity of bromine-containing acetonitriles was greater than that of DCAN.

Binding of DCAN to Polynucleotides and DNA

The binding of [^{14}C]-DCAN to polynucleotides, including polyadenylic acid (Poly A), polyguanylic acid (Poly G), and DNA, was determined. [1-^{14}C]-DCAN was incubated with the polynucleotides and hydrolyzed as previously described (*4*). Strong acid hydrolysis (1 N HCl at 100°C for 1 hr) of [^{14}C]-DCAN-bound Poly A resulted in the appearance of adducts as peaks III and IV in the high pressure liquid chromatography (HPLC) elution profile from a Whatman Partisil 10 Magnum 9 SCX column (Fig. 3*A*). Strong acid hydrolysis of [^{14}C]-DCAN-bound Poly G resulted in a radioactive peak (peak III) that was eluted in a region of the profile similar to peak III of the [^{14}C]-DCAN-bound Poly A (Fig. 3*B*). Dilute acid hydrolysis (0.1 N HCl at 70°C for 30 min) of [^{14}C]-DCAN-bound DNA resulted in a major peak (peak IV) in the region of the elution profile of Peak IV from DCAN-bound Poly A and a minor peak (peak II; Fig. 3*C*). Strong acid hydrolysis of DCAN-bound DNA resulted in the disappearance of peak II with the occurrence of peaks III and IV in the region similar to peaks III and IV of the elution profile of DCAN-bound Poly A (Fig. 3*D*). Thus, the adduct present in peak IV of the elution profile of DCAN-bound Poly A appears also to be formed when DCAN binds to DNA. In conclusion, DCAN has been shown to bind directly to DNA with the formation of an adduct to adenosine and possibly other sites in the DNA. However, oral administration of [^{14}C]-DBAN or [^{14}C]-DCAN did not result in any detectable adduct formation in rat liver (results not shown).

Cancer Initiation Bioassay

An initiation-promotion bioassay in rat liver, the rat liver foci bioassay, is being developed for determining the tumor-initiating activity of chemical carcinogens (*8*). Briefly, the test substance is administered to partially hepatectomized rats followed 1 week later by the admin-

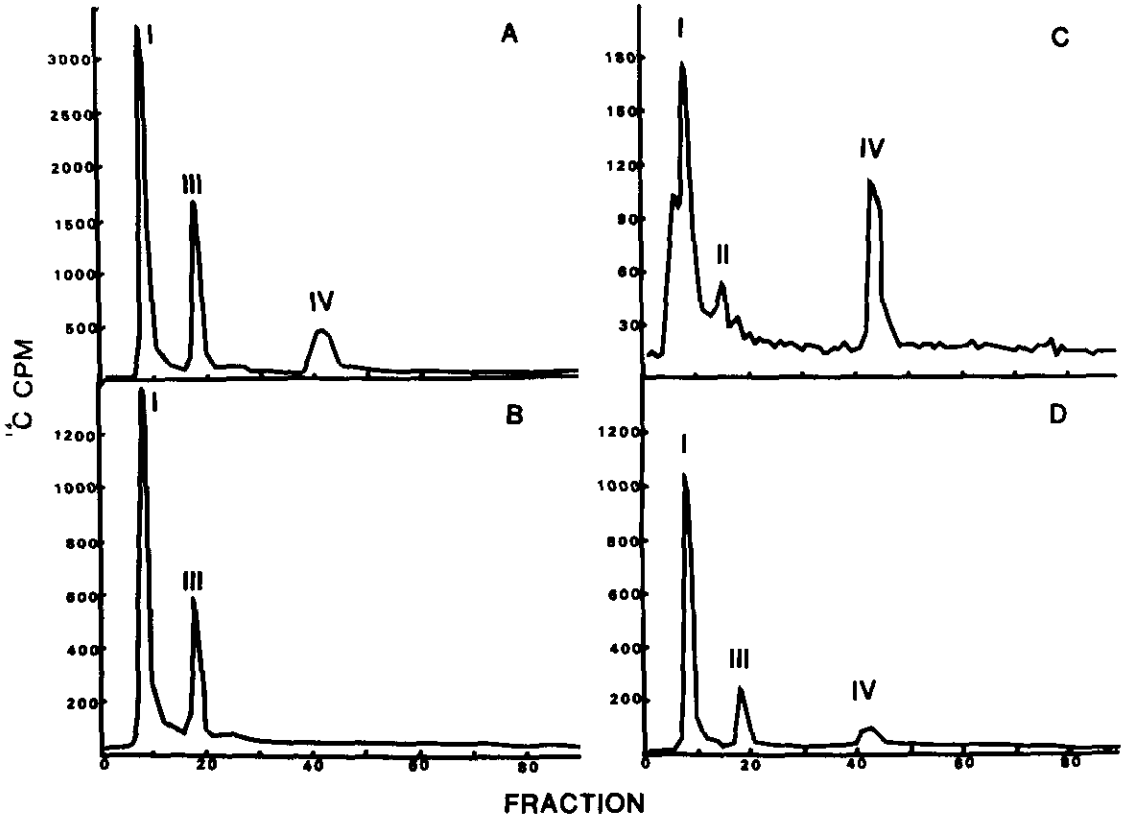

FIGURE 3. HPLC elution profile of hydrolyzed Poly A-, Poly G-, or DNA-containing bound [^{14}C]-DCAN: (*A*) Strong acid hydrolysis (1 N HCl at 100°C for 1 hr) of [^{14}C]-DCAN-bound Poly A; (*B*) strong acid hydrolysis of [^{14}C]-DCAN-bound Poly G; (*C*) dilute acid hydrolysis (0.1 N HCl at 70°C for 30 min) of [^{14}C]-DCAN-bound DNA; (*D*) strong acid hydrolysis of [^{14}C]-DCAN-bound DNA. [^{14}C]-DCAN was incubated with either Poly A, Poly G, or calf thymus DNA. The hydrolyzates were applied to an HPLC column (Whatman Partisil 10 Magnum 9 SCX column, (9.4 mm × 250 mm) and eluted with a mobile phase consisting of 0.025 M ammonium phosphate, pH 4.0, for 10 min, followed by a linear changeover in 15 min to 0.20 M ammonium phosphate, pH 4.0, in 10% methanol. The flow rate was 4.0 mL/min, and 2.0 mL fractions were collected.

Table 3. Tumor-initiating activity of haloacetonitriles in the rat liver foci bioassay.[a]

Chemical	Dose, mmole/kg	GGT foci/cm^2[b]
CAN	1.0	0.46 ± 0.20 (10)
DBAN	2.0	1.00 ± 0.29 (6)
DCAN	2.0	0.2 ± 0.12 (5)
TCAN	2.0	0.0 ± 0.0 (7)
Tricaprylin (vehicle)	—	0.31 ± 0.31 (9)
Diethylnitrosamine	0.3	9.51 ± 2.34 (9)

[a] The test substance was administered to Fischer-344 rats 24 hr after a 2/3 partial hepatectomy. One week after the operation, the animals started to receive 500 ppm sodium phenobarbital for a total of 8 weeks. One week after the cessation of treatment with phenobarbital, the animals were sacrificed and the liver was scored for the occurrence of GGT foci.

[b] Mean ± SE. The number of animals is presented in parentheses.

istration of 500 ppm sodium phenobarbital in the drinking water for a total of 8 weeks. The partial hepatectomy is used to increase the sensitivity of the assay by inducting DNA replication during the binding of the test substance to DNA (*9–11*). The sodium phenobarbital is used to promote the appearance of preneoplastic and neoplastic lesions (*12,13*). The putative preneoplastic lesion, foci of hepatocytes containing γ-glutamyltranspeptidase (GGT) activity, is used to indicate the occurrence of carcinogenesis initiation. Hepatocytes of rat liver lack GGT activity, whereas tumors contain GGT activity, especially when phenobarbital is used as a promoter (*12,13*). The initiation of hepatocarcinogenesis is believed to result in the occurrence of the morphologically altered cells that progress through clonal expansion into foci of altered hepatocytes (*12,14*). These foci of altered hepatocytes can, after further alteration (rare events), progress to neoplastic lesions. GGT foci are one type of altered foci used to indicate the occurrence of cancer initiation (*12,14*).

Orally administered HAN were tested in rat liver foci bioassay for tumor-initiating activity (Table 3). None of the four HAN induced GGT foci in rat liver. Diethylnitrosamine, which was used as a positive control, did induce GGT foci. Thus, the HAN appear to lack tumor-initiating activity in rat liver.

Conclusions

The HAN have been found in chlorinated drinking water (*3,15*) and have been shown to be formed in the stomach of rats after gavage administration of an aqueous solution of chlorine (*16*). Bromide in an aqueous solution of chlorine is oxidized to hypobromite (*17*). Thus, chlorination of drinking water that contains bromide could result in the formation of chlorine- and bromine-containing HAN. The presence of HAN in drinking water or the production of HAN in the gastrointestinal tract after consumption of drinking water containing residue chlorine might pose a human health hazard.

Simmon et al. (*3*) have reported that DCAN is mutagenic in the Ames Salmonella assay. The HAN also induce sister-chromatid exchange in Chinese hamster ovary (CHO) cells (*18*). In this paper, we report that the HAN possessed direct-acting alkylating activity, inhibited DMN-DM activity *in vitro*, bound *in vitro* to polynucleotides including DNA, induced DNA strand breaks in cultured human lymphoblastic cells of T-cell origin, were excreted as thiocyanates in the urine, and did not exhibit tumor-initiating activity in the rat liver foci bioassay. The results of these chemical and biological evalutions of the HAN are summarized in Table 1. No correlation was observed between their alkylation potential measured by their binding to NBP and their ability to produce DNA strand breaks in cultured cells or their ability to inhibit microsomal DMN-DM activity.

A nucleophilic reagent can react with HAN either by displacing a halogen atom or by attacking the carbon atom of the cyano group that has a partial positive charge resulting from the resonance:

$$R-C\equiv N \leftrightarrow R-\overset{+}{C}=\overset{-}{N}$$

NBP probably reacts with HAN through displacement of halogen, a conjecture derived from the similarity of results between HAN and alkyl halides (*5*). Under similar conditions, amyl cyanide did not react with NBP (*5*). An electron-withdrawing substituent on the α-carbon increases the reaction rate of nucleophilic addition toward the cyano group (*18*). Thus, TCAN would be the most reactive among the HAN toward nucleophilic addition on the cyano group in contrast to the nucleophilic substitution of halogen. The contrasting results between the binding of HAN to NBP and their ability to cause DNA strand breaks or to inhibit DMN-DM activity could be the result of NBP reaction occurring by displacement of halogen, whereas the other effects occur both by displacement of halogen and by attack on the cyano group.

The HAN are absorbed systemically and are converted to toxic metabolites as demonstrated by their excretion as thiocyanates and their inhibition of DMN-DM activity in rat liver. However, the HAN failed to produce any detectable amounts of DNA adducts in rat liver following oral administration, failed to initiate GGT foci in the rat liver foci bioassay, and failed to induce micronuclei in polychromatic erythrocytes of bone marrow of CD-1 mice (*19*). This inability to demonstrate a systemic activity for HAN could be caused by the failure of these compounds to reach the target organ and/or to their rapid *in vivo* detoxification. The tumor-initiating activity of HAN in mouse skin when applied topically indicates that a carcinogenic hazard may exist at the site of application. Thus, if HAN which are present in drinking water or which are formed in the gastrointestinal tract after consumption of drinking water containing a residue of chlorine represent a carcinogenic hazard, the site of the hazard would most likely be limited to the gastrointestinal tract. Further investigations are required to determine whether the HAN are gastrointestinal carcinogens.

The research described in this article has been reviewed by the Health Effects Research Laboratory and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

This paper is being presented in honor of the retirement of Mr. Leland J. McCabe from a long and very successful government service. His guidance and support as Division Director were instrumental in the successful completion of the research described in this paper.

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