TLC-Fractionation and GC/MS Characterization of Mutagenic Substances in Chlorinated 4-Alkylphenol Solutions

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Summary

The diethyl ether extracts from aqueous 4-alkylphenol solutions after treatment with chlorine were mutagenic to the Ames *Salmonella* tester strains TA98 and TA100 in the absence of rat liver homogenate. Gas chromatography/mass spectrometry (GC/MS) showed the occurrence of chlorinated products in the extracts; chloro-4-alkylphenols, chloro-4-alkylquinones and chlorinated 4-alkylphenol dimers. The diethyl ether extracts were fractionated into several fractions by polyamide thin-layer chromatography (TLC). The fractionated components were examined for mutagenicity by means of Ames assay, and were identified by GC/MS. TLC fractionation of the extracts revealed that two components present in the extracts are mutagenic. GC/MS analyses indicated the presence of 2,6-dichloro-4-hydroxy-4-alkylcyclohexa-2,5-dien-1-ones as the major mutagen and chlorinated 4-alkylphenol dimers as minor mutagenic compounds in the chlorinated 4-alkylphenol solutions.

Key words: water chlorination, 4-alkylphenol, mutagenicity, chloroalkylsemiquinone

INTRODUCTION

Mutagenic and carcinogenic organic compounds found in source water¹⁾ and drinking water²⁾ have recently caused concern as to their potential effects on human health. Mutagenicity in source waters has generally been attributed to contamination by industrial waste or agricultural run-off, and to a lesser extent to naturally occurring substances. In comparison studies of raw water vs. finished water, Glatz et al.³⁾ and Maruoka and Yamanaka⁴⁾ suggested that chlorination may play a major role in the production of organic muta-

gens in potable water. Subsequent laboratory studies by Cheh et al.⁵⁾ in which a drinking water treatment process was simulated, clearly demonstrated that non-volatile mutagens were produced by chlorine disinfection. Several volatile compounds such as chloroacetones and chloropropenal, and chlorinated hydroxyfuranone (MX), exhibiting mutagenicity have been identified to be present in chlorinated water^{6,7)}, but many of the non-volatile mutagenic substances have not been fully characterized.

One class of compounds recognized as important pollutants in aquatic environment is phenols because of

off-favor in water and their high toxicity. Therefore, it seems that mutagens are formed as a result of the reaction of chlorine with phenolic compounds that may be present in the water sources. Direct support for this hypothesis has been provided by Rapson et al.⁸⁾ and by our recent studies which demonstrated that phenols react with chlorine to form direct-acting mutagenic products⁹⁻¹³⁾. We now present more detailed finding on the characterization of the mutagens formed by the reactions of chlorine with 4-alkylphenols in aqueous solution. 4-Alkylphenols were chosen in this work because these compounds have been used as intermediates in the production of important industrial chemicals and also identified as being present in aquatic samples¹²⁾.

EXPERIMENTAL

Chemicals

4-Alkylphenols (C₁-C₈), 2-chloro-4-alkylphenols, 2,6-dichloro-4-alkylphenols, Irgasan DP 300 (as a typical chlorinated phenoxyphenol) and trichloroacetic acid used for product identification and mutagenicity tests were commercially products. Standard solutions of these compounds both alone and as mixtures were prepared by dissolving the compounds in methanol and subsequent dilution. Chlorinated aqueous solution was prepared by diluting sodium hypochlorite solution (ca. 10 % available Cl) (Nacalai Tesque, Kyoto Japan) with 0.1 M potassium dihydrogen phosphate/sodium tetraborate decahydrate solution. The hypochlorite concentrations were determined by iodometric titration. Polyamide 11 F₂₅₄ (20×20 cm, thickness 0.15 mm) precoated thin-layer chromatographic (TLC) plates (Merck, Darmstadt, Germany) were used to fractionate the diethyl ether extracts.

Aqueous chlorination of 4-alkylphenol and extraction of reaction mixture

Individual aqueous 4-alkylphenol solutions (0.5 mmol/ℓ) were treated with 5 equiv. of chlorine (as HOCl) at 20 °C for 1 h at pH 5. The unreacted chlorine was removed by addition of sodium thiosulfite solution. The reaction mixture was then acidified to pH 2 with 6 M hydrochloric acid before extraction with two 200-mℓ portions of diethyl ether. The extracts were dried over anhydrous sodium sulfate and concentrated under vacuum at 40 °C to volumes suitable for polyamide TLC, GC and GC/MS analyses. Part of the diethyl ether extract was resuspended in 1 mℓ of dimethyl sulfoxide (DMSO) for mutagenicity assays.

Fractionation of mutagenic extracts by TLC

Individual diethyl ether extracts (about 50 mg) were fractionated into several fractions by TLC on polyamide plates using chloroform as a developing solvent. The detection of spots on the plates was performed by UV irradiation. The plates were also sprayed with 0.5 % bromocresol green solution in order to detect acidic products. The separated zones were scraped off by using special recovery tubes (Wako, Osaka, Japan) and the adsorbed substances were eluted with diethyl ether. The recoveries (% w/w) of individual fractions were calculated as [(amount of sample recovered)/amount of sample applied]×100.

Product resolution and characterization

A Hitachi Model 263-30 gas chromatograph equipped with a flame ionization detector and a 2 m×3 mm i.d. glass column packed with 2 % OV-1 on Uniport HP (60-80 mesh) was programmed from 100 to 260 °C. The injector and detector temperatures were 260 °C. The helium gas flow-rate was 30 mℓ/min. A Hitachi D-2500 chromato-integrator was used to determine the retention times and peak areas on the chromatograms.

A Hitachi M-80 combined mass spectrometer-gas chromatograph with a Hitachi M-003 data processing system was used for the qualitative analyses of samples under the following conditions: ion source temperature, 250 °C; trap current, 70 μA; and electron energy, 70 eV. The column and chromatographic conditions were the same as described above.

Mutagenicity tests

The mutagenicity of the samples was tested according to the method of Ames et al.¹⁵⁾ with minor modifications. Liver homogenates (S9) was prepared from male Sprague-Dawley rats, which had been pretreated with phenobarbital and 5,6-benzoflavanone to activate enzymes. Addition of the S9 mix was conducted to confirm whether test compounds were indirect-acting mutagens which required mammalian metabolic activation. *Salmonella typhimurium* strains TA98 and TA100 were used throughout the experiments. The samples were dissolved in DMSO and preincubated with a tester strain at 37 °C for 20 min (prior plating) with and without S9 mix. A buffer was used when S9 mix was not employed. After addition of the test sam-

ples, the plates were incubated at 37 °C for 2 days. The assay was performed in triplicate for each sample. The mutagenic activity is expressed as the mean value of revertants.

RESULTS AND DISCUSSION

Mutagenic potential formation of 4-alkylphenols during aqueous disinfection with chlorine

Treatment with chlorine of 4-alkylphenols in aque-

ous solutions and subsequent mutagenicity assays of these diethyl ether extracts were performed to evaluate the mutagenic potential formation of these compounds. Table 1 is a summary showing the experimental conditions for aqueous chlorination of 4-alkylphenols and the results of Ames tests for the diethyl ether extracts. Figure 1 shows typical examples of the actual mutagenic responses of extracts from chlorine-treated 4-alkylphenol solutions on *Salmonella typhimurium* TA100.

Table 1 Mutagenic potential formation (MPF) of 4-alkylphenol solutions by chlorination

Expt. No.	Compound examined		HOCl		Reaction Time (h)	MPF in TA98		MPF in TA100	
	Name	Conc. (mM)	Conc. (mM)	Solution pH		net revertants/nmol -S9	+S9	net revertants/nmol -S9	+S9
1	4-Methylphenol	0.5	2.5	5	1	1.23	0.32	23.78	3.20
2	4-Ethylphenol	0.5	2.5	5	1	0.82	0.21	15.80	2.12
3	4-Propylphenol	0.5	2.5	5	1	0.79	0.20	12.78	1.65
4	4-Butylphenol	0.5	2.5	5	1	0.91	0.23	11.25	1.46
5	4-Pentylphenol	0.5	2.5	5	1	0.67	0.17	10.87	1.41
6	4-Hexylphenol	0.5	2.5	5	1	0.55	0.14	9.95	1.29
7	4-Heptylphenol	0.5	2.5	5	1	0.30	0.08	7.50	0.97
8	4-Octylphenol	0.5	2.5	5	1	0.20	0.05	4.00	0.52
9	4-Nonylphenol	0.5	2.5	5	1	0.10	0.03	2.50	0.32
Positive control		2-Nitrofluorene				31.43	-	-	-
		Sodium Azide				-	-	33.00	-
		Benzo (a) pyrene				-	28.00	-	37.00

Fig. 1 Mutagenicity of diethyl ether extracts of aqueous 4-alkylphenol solutions after treatment with hypochlorous acid (5 equivalents of HOCl per mole of compound) at pH 5 for 1 h, as measured with *S. Typhimurium* TA100 in the absence of S9 mix

None of the 4-alkylphenols before aqueous chlorination were mutagenic in the TA98 and TA100 strains with or without S9 mix. Aqueous chlorination, however, generated mutagens from non-mutagenic parent compounds. Greater mutagenic potential formation on the TA100 strain without S9 mix were observed for the 4-alkylphenol-hypochlorite reactions, as compared with those detected on the TA98 strain (Table 1). An increase in the alkyl chain length in the phenol ring decreased the mutagenic potential formation when 4-alkylphenols were treated with chlorine in an acidic aqueous solution of pH 5 for 1 h. A weak mutagenic potential formation on the both tester strains without S9 mix was observed even for the nonylphenol-chlorine reaction.

A previous study¹³⁾ revealed that treatment with a high chlorine dose (20 equiv. of HOCl per mole of compound) of 4-methylphenol in neutral aqueous solution (pH 7) produces a series of highly chlorinated compounds. These were chlorinated 4-methylphenols, chlorohydroxylated 4-methylquinones and polychlorinated 4-methylphenol dimers. Among these chlorinated com-

pounds, polychlorinated 4-methylphenol dimers have also been shown to be mutagenic towards test strain TA100 without S9 mix. However, our recent studies^{9,11)} demonstrated that aqueous chlorination of 4-methylphenol produces the mutagen (2,6-dichlorotolquinol) even at a low chlorine dose (5 equiv. of HOCl per mole of compound) in an acidic solution of pH 5. Since mutagenic substances occurring in the other 4-alkylphenol-hypochlorite reactions were not yet isolated and characterized, further GC/MS studies combined with TLC fractionation of the mutagenic diethyl ether extracts were performed again in this work.

TLC fractionation of mutagenic diethyl ether extracts

A preliminary fractionation of the mutagenic diethyl ether extracts from the chlorine-treated 4-methyl-, ethyl-, propyl- and butylphenol solutions was performed by TLC using polyamide plates. The TLC results for the original extracts and mutagenicity tests with strain TA100 in the absence of S9 mix are presented in Figs. 2 and 3. When the original diethyl

Fig. 2 Fractionation of mutagenic diethyl ether extracts of chlorine-treated 4-alkylphenol solutions by polyamide TLC

For TLC conditions, see Experimental

ether extracts were fractionated on polyamide plates, the recovery in the fractions was approximately 60~75 % (w/w) of the amount of samples applied, while the mutagenicity recovered from the plates was about 50~60 % of the over all activity applied. These results indicate that mutagenic substances in the extracts are converted into non-mutagenic materials during the TLC fractionations¹⁴. In addition, most of the mutagenic materials were found to be concentrated in each fraction 3 on the polyamide plates (Figs. 2 and 3). This suggests that the mutagenic materials in the extracts have intermediate polarity.

The dose-response curves of the typical TLC-fractionated samples are shown in Fig. 3. Among these components, each fraction 3 showed the strongest mutagenic response, and their mutagenic potentials were approximately 10-24 revertants/nmol, dependent on the alkyl chain length in the phenol ring. These potentials were nearly the same as those reported for 1,1,3-trichloroacetone, 2-chloropropenal and sodium azide as be-

ing used for positive reagent in Ames assay, but much lower than that found for chlorinated hydroxyfuranone (MX), which have been cited as important mutagens in kraft chlorination effluents⁶. Fractions 6 and 7 also exhibited mutagenicity, but the activity was very low as compared with those of each fraction 3. Most of the mutagenicity of original extracts obtained from the chlorinated 4-alkylphenol solutions were thus accounted for by each fraction 3.

GC and GC/MS studies of the TLC fractions

In order to obtain further information on the mutagenic components present in the diethyl ether extracts of chlorine-treated 4-alkylphenol solutions, the components (fractions 1-7) obtained by polyamide TLC were investigated by means of GC and GC/MS. Fig. 4 shows the gas chromatograms (flame ionization detection) of the original extracts and fractions 3, 4 and 7. 2-Chloro-4-alkylphenols and 2,6-dichloro-4-alkylphenols, corresponding peaks 1 and 2, in Fig. 4 were distrib-

Fig. 3 Mutagenicity of the original extracts of chlorine-treated 4-alkylphenol solutions and polyamide TLC fractions 3 and 7 against *S. typhimurium* TA100 without S9 mix

Each point on the dose-response curves represents the average of triplicate determination

—○— = original extracts; —△— = fraction 3; —□— = fraction 7

Fig. 4 Gas chromatograms (flame ionization detection) of the original extracts of 4-alkylphenols and polyamide TLC fractions 3, 4 and 7

For main compounds, see text; for GC conditions, see EXPERIMENTAL

uted mainly in fraction 4, but these compounds exhibited no mutagenicity response. Polychlorinated 4-alkylphenol dimers were also distributed in fractions 6 and 7, increasing amount and mutagenicity. Fraction 7, with a weak mutagenicity response, consisted of a mixture of di-, tri- and tetrachlorinated 4-alkylphenol dimers. These compounds have been shown previously^{13,15,16} to be the mutagenic materials in the extracts from the 4-alkylphenol solutions after treatment with a high chlorine dose at pH 7 for 1 h.

Each fraction 3 in Fig. 2, with the highest mutagenic response (Fig. 3), consisted of unknown compounds corresponding to the peak 3 in Fig. 4, and their purity were over 90 % by GC determinations with flame ionization detection. The gas chromatograms in Fig. 4 also show that these compounds are the major component in the original extracts. Therefore, it seems that these compounds, formed by the reactions of 4-alkylphenols with chlorine in an acidic aqueous solutions, contribute to the mutagenicity of the chlorinated 4-alkylphenol solutions.

Figure 5 shows the mass spectra of unknown compounds that correspond to each TLC fraction 3 in Fig. 2 and peak 3 in Fig. 4, with the highest mutagenic re-

sponse, and their mass spectrometric interpretations are also presented in Table 2. The unknown compounds gave the molecular ions (M^+) at m/z 192 for the 4-methyl-, 206 for the 4-ethyl-, 220 for the 4-propyl- and 234 for the 4-butylphenol-hypochlorite reaction products, respectively, with two chlorine atoms. The most abundant fragment ions at m/z 177 (M^+ -alkyl) and m/z 157 (M^+ -Cl for the methyl-), 171 (M^+ -Cl for the ethyl-), 185 (M^+ -Cl for the propyl-) and 199 (M^+ -Cl for the butylphenol reaction product), respectively, were also observed in each mass spectrum in Fig. 5. These findings indicate the occurrence of $C_7H_5Cl_2O_2$, $C_8H_7Cl_2O_2$, $C_9H_9Cl_2O_2$ and $C_{10}H_{11}Cl_2O_2$ in the highest mutagenic fraction 3. On the basis of these MS interpretations for TLC fraction 3 and that obtained previously⁹, the mutagenic compounds present in the fraction 3 may be 2,6-dichloro-4-hydroxy-4-alkylcyclohexa-2,5-dien-1-ones.

CONCLUSIONS

The compounds responsible for the mutagenic response could be completely isolated from the diethyl ether-extractable products of the reactions of 4-alkylphenols with hypochlorous acid in acidic aqueous solu-

Fig. 5 Mass spectra (EI-MS) of the unknown compounds corresponding to the peak 3 in the chromatogram in Fig. 4 and to the fraction 3 in the thin-layer chromatogram in Fig. 2

Table 2 Mass spectrometric (EI-MS) characterization of 2,6-dichloro-4-hydroxy-4-alkylcyclohexa-2,5-dien-1-ones (chloroalkylsemiquinones) occurring in the mutagenic diethyl ether extracts from chloro-treated 4-alkylphenol solutions (pH 5)

Compound tested	EI-MS fragmentation (EI, 70 eV)	
	m/z [fragment ion, relative intensity (%)]	
1. Chloromethylsemiquinone	192 (M ⁺ , 5), 177 (M ⁺ - CH ₃ , 70), 157 (M ⁺ - Cl, 100), 149 (M ⁺ - CH ₃ -CO, 44), 129 (M ⁺ - Cl-CO, 55), 111 (M ⁺ - ?, 30), 85 (M ⁺ - CH ₃ -Cl-CO-COH, 56)	
2. Chloroethylsemiquinone	206 (M ⁺ , 0.8), 177 (M ⁺ - C ₂ H ₅ , 100), 171 (M ⁺ - Cl, 72), 149 (M ⁺ - C ₂ H ₅ -CO, 44), 114 (M ⁺ - C ₂ H ₅ -Cl-CO, 39), 85 (M ⁺ - C ₂ H ₅ -Cl-CO-COH, 60)	
3. Chloropropylsemiquinone	220 (M ⁺ , 0.8), 185 (M ⁺ - Cl, 30), 178 (M ⁺ - C ₃ H ₇ , 100), 149 (M ⁺ - C ₃ H ₇ -CO, 27), 114 (M ⁺ - C ₃ H ₇ -Cl-CO, 31), 85 (M ⁺ - C ₃ H ₇ -Cl-CO-COH, 36)	
4. Chlorobutylsemiquinone	234 (M ⁺ , 0.5), 199 (M ⁺ - Cl, 11), 178 (M ⁺ - C ₄ H ₉ , 100), 163 (M ⁺ - ?, 12), 149 (M ⁺ - C ₄ H ₉ -CO, 10), 114 (M ⁺ - C ₄ H ₉ -Cl-CO, 11), 85 (M ⁺ - C ₄ H ₉ -Cl-CO-COH, 22)	

tions. The compounds isolated was mutagenic in the TA100 strain without mammalian metabolic activation, and their mutagenic potentials were ranged from 2.5 for the 4-nonylphenol- to 24 revertants per nmol for the 4-methylphenol-hypochlorite reaction product. GC/MS studies of the mutagenic components isolated suggested that these compounds are probably 2,6-dichloro-4-hydroxy-4-alkylcyclohexa-2,5-dien-1-ones (chlorinated semiquinones) which have yet to be identified. Therefore, subsequent investigations will be conducted to identify and determine the chlorinated semiquinones in chlorine-treated waters, such as pulp mill effluents, industrial wastewater and drinking water.

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