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Identification of co-mutagenic chlorinated harmans in final effluent from a sewage treatment plant

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Abstract

Harman (1-methyl-9*H*-pyrido[3,4-*b*]indole) reacted readily with sodium hypochlorite in an aqueous medium to give the mono-chlorinated derivatives, which reportedly have greater co-mutagenic activity than harman in the presence of *o*-toluidine toward *Salmonella typhimurium* TA 98 with S9 mix. Mono-chlorinated harmans were detected by concentration using blue rayon (BR) and GC/MS analysis in the final effluent from a sewage treatment plant in Shizuoka, Japan. The amounts adsorbed for 24 h were 1–45 ng/g BR for mono-chlorinated harman and 110–730 ng/g BR for harman.

Keywords: Harman; Co-mutagenicity; Disinfection by-product; Blue rayon

1. Introduction

Harman (1-methyl-9*H*-pyrido[3,4-*b*]indole) and norharman (9*H*-pyrido[3,4-*b*]indole) are present in alcoholic beverages, like beer, wine, sake and whisky, in cigarette smoke, and in fried meat and fish [1–5]. Although harman and norharman, β-carbolines, have skeletons similar to those of mutagenic and carcinogenic heterocyclic aromatic amines, it has been reported that they themselves are not mutagenic to *Salmonella typhimurium* TA98, either with or without S9 mix. However, they became mutagenic toward *S. typhimurium* TA98 with S9 mix when incubated with non-mutagenic aromatic amines, such as aniline or *o*-toluidine. Accordingly, harman and norharman have been called "co-mutagens" [6–9]. Recently, we reported that such β-carbolines can be easily chlorinated with sodium hypochlorite and the chlorinated products show 20 times more potent co-mutagenic activity than the β-carbolines themselves in Ames test using *S. typhimurium* TA98 in the presence of *o*-toluidine [10].

It is generally difficult to analyse water pollutants because they are present in only minute concentrations. Therefore, approaches to concentrate pollutants in river water are very important. Frequently used methods include extraction with organic solvents, a variety of XAD resins, and Sep-pack cartridges. In addition, blue cotton and blue rayon, which consist of cotton and rayon covalently bonded to the blue pigment copper phthalocyanine trisulfonate, can specifically adsorb multicyclic planar compounds, as reported by Hayatsu [11].

In the present study, we attempted to isolate chlorinated harman that had been concentrated using blue rayon from final effluent of a sewage treatment plant, where harman was found to be present at higher level

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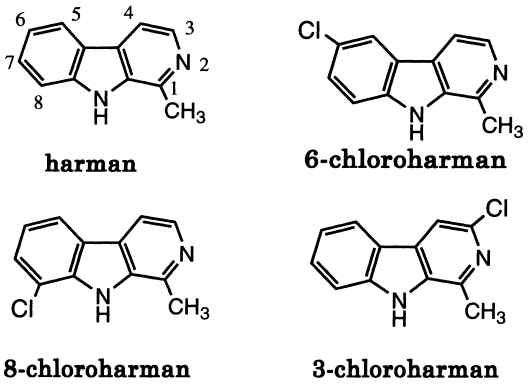

Fig. 1. Chemical structures of harman and chloroharman.

than norharman. We describe here the identification of three chlorinated harmans (Fig. 1) isolated by GC/MS analysis.

2. Experimental

2.1. Materials

Harman was purchased from Aldrich Chemical Co. Inc. Both 6- and 8-chloroharmans were synthesised by chlorination of harman and purified by the same method as reported previously [10]. The solvents used were of pesticide analysis grade and sodium hypochlorite was purchased from Wako Pure Chemicals Industry Ltd. Blue rayon was obtained from Funakoshi Co. Ltd., Tokyo. The solid-phase extraction disk SDB-XD was from Sumitomo 3 M, Ltd. The silica gel column was prepared by placing 5 g of silica S-60 (Merck, Japan) in a glass tube (10 mm i.d. × 300 mm). Water was treated with a Milli-Q water purification system after distillation and then purified by an activated carbon column. ^{1}H-NMR spectra of solutions in chloroform-*d* were taken with a JEOL JNM-GSX270 (^{1}H: 270 MHz) Fourier transform spectrometer. The following abbreviations are used: s = singlet, d = doublet, t = triplet, m = multiplet, br = broad. Chemical shifts are shown in ppm using tetramethylsilane as an internal standard.

2.2. GC/MS apparatus and operating conditions

Gas chromatography/mass spectrometry (GC/MS): Hewlett Packard HP-5890 II gas chromatograph and Hewlett Packard HP-5971A mass spectrometer. The GC/MS conditions were as follows: column, HP-5 trace analysis (5% diphenyl and 95% dimethylarylenesiloxane, coating film thickness, 0.1 μm; 0.25 mm i.d. × 30 m, Hewlett Packard Co.); injection mode, splitless; injection temperature, 250°C; column head pressure, 7 psi; column temperature, 70–250°C; program rate, 10°C/min; ionising voltage, 70 eV. MS measurement was performed in scan mode for a qualitative analysis and in the selected ion monitoring (SIM) mode for a quantitative analysis. The monitored ions were the peaks at *m/z* 154, 181 and 182 for harman and *m/z* 181, 216 and 218 for mono-chlorinated harmans.

2.3. Chlorination of harman with sodium hypochlorite in aqueous medium

A solution of sodium hypochlorite was diluted with pure water to 10 mg/l of available chlorine, and was adjusted to pH 4 with 0.1 M hydrochloric acid. To 20 ml of this solution was added 400 μl of harman solution (1 mg/ml acetone solution) and the mixture was allowed to stand for 5 min at room temperature. Sodium thiosulfate was added and the mixture was adjusted to pH 8 with 0.2 M sodium hydroxide. The reaction products were extracted twice with 2 ml of dichloromethane. The extract was dried over anhydrous sodium sulfate and was subjected to GC/MS analysis.

3-Chloroharman that had been newly produced in aqueous medium was isolated by a method similar to that described previously [10] after a large-scale reaction.

3-Chloro-1-methyl-9H-pyrido[3,4-b]indole (3-chloroharman): mp 220–222°C. HRMS *m/z*: 216.0429 (M^+), calcd for $C_{12}H_9ClN_2$, 216.0454. ^{1}H-NMR ($CDCl_3$) δ: 2.87 (1H, s, CH_3), 7.23 (1H, dt, J = 8.1, 1.0 Hz, 6-H), 7.50 (1H, d, J = 8.1 Hz, 8-H), 7.56 (1H, dt, J = 8.1, 1.0 Hz, 7-H), 7.80 (1H, s, 4-H), 8.05 (1H, d, J = 8.1 Hz, 5-H). 8.4 (1H, br, NH).

2.4. Determination of harman in final effluent from a sewage treatment plant by extraction with a solid-phase disk

A solid-phase extraction disk (SDB-XD 47 mm Ø) was washed with 5 ml of dichloromethane, 5 ml of

methanol and then 20 ml of pure water. Water samples (3 l) were extracted with three disks. Harman extracted on the disks was eluted with dichloromethane (4 ml, two times). The combined solution was dried over anhydrous sodium sulfate, and concentrated on a rotary evaporator to about 1 ml and further concentrated to 0.5 ml under a nitrogen stream. Harman was determined by GC/MS analysis.

2.5. Determination of harman and chlorinated harmans in effluent from sewage treatment plant by concentration with blue rayon

Three wire net bags of blue rayon (5 g per bag) were hung in a final effluent from sewage treatment plant for 24 h. The three samples of blue rayon were combined and washed five times with 1 l of pure water. Adsorbed materials were extracted three times by shaking the blue rayon in 300 ml of methanol/ammonia water (50/1, v/v). The combined extracts were concentrated to about 20 ml under reduced pressure. To this solution was added 100 ml of pure water and 10 g of sodium chloride, and the mixture was then extracted three times with 30 ml of dichloromethane. The dichloromethane layers were combined, dried over anhydrous sodium sulfate, concentrated to about 1 ml, and then applied to a silica gel column. After eluting with 40 ml of hexane and 20 ml of 10% ethyl acetate in hexane, the fractions eluted with 20 ml of ethyl acetate and 30 ml of acetone were concentrated to 1 ml and then were subjected to GC/MS analysis.

3. Results and discussion

3.1. Chlorination of harman with sodium hypochlorite in an aqueous medium and GC/MS analysis of the products

Since preliminary experiments showed that the chlorination of harman occurred rapidly with a low concentration of sodium hypochlorite, the reactions of harman (400 μg) were carried out by standing for 5 min in 20 ml of sodium hypochlorite solutions (chlorine, 10 mg/l). The GC/MS chromatogram of the extract from the reaction mixture is shown in Fig. 2. The peak of unreacted harman is D and there are eight new peaks (A, B, C, E, F, G, H and I) for the products. The MS spectra of peaks A, D and G are shown in Fig. 2.

In the MS spectrum of peak G (Fig. 2), the molecular ion peaks appear at *m/z* 216 and 218 with a relative abundance of about 10:3, which suggests that they are due to mono-chlorinated harman. The MS spectra of peaks E and F also showed the same molecular ion peaks at *m/z* 216 and 218 and similar fragment patterns. These peaks may be due to isomers of mono-chlorinated harman observed as peak G.

Peaks E and F were identified to be those of 8- and 6-chloroharman, respectively, by comparison with the GC retention times of synthetic compounds described previously [10]. Peak G was found to be due to the third isomer of mono-chlorinated harman. The isomer was isolated and determined to be 3-chloroharman by ^{1}H-NMR analysis.

Peaks H and I showed similar MS spectra and both had molecular ion peaks appeared at *m/z* 250, 252 and 254 (relative abundance = 10:6:1), suggesting di-chlorinated harman. They were assumed to be the positional isomers, but were not identified.

The MS spectrum of peak A shown in Fig. 2 indicated that this compound is a mono-chlorinated compound. However, this is not a harman derivative. The structure was assumed to be a chlorinated derivative of acetyl indole derived from harman. The MS spectra of peaks B and C also suggested that they have the same skeleton. Further structural determination and the formation mechanism of these compounds are under investigation.

3.2. Identification of harman and chlorinated harmans in final effluent from a sewage treatment plant

Three wire net bags that contained blue rayon (5 g per bag) were hung in final effluent from a sewage treatment plant in Shizuoka, Japan, for 24 h. Since the extracts from final effluent contained various compounds, they were purified using a silica gel column. The fractions including harman and chlorinated harmans were concentrated and subjected to GC/MS analysis. Their SIM chromatograms are shown in Fig. 3. The fraction eluted with ethyl acetate contained 3-chloroharman and that eluted with acetone contained harman and 6- and 8-chloroharman.

The quantitative results are summarised in Table 1. Harman was detected in every measurement and

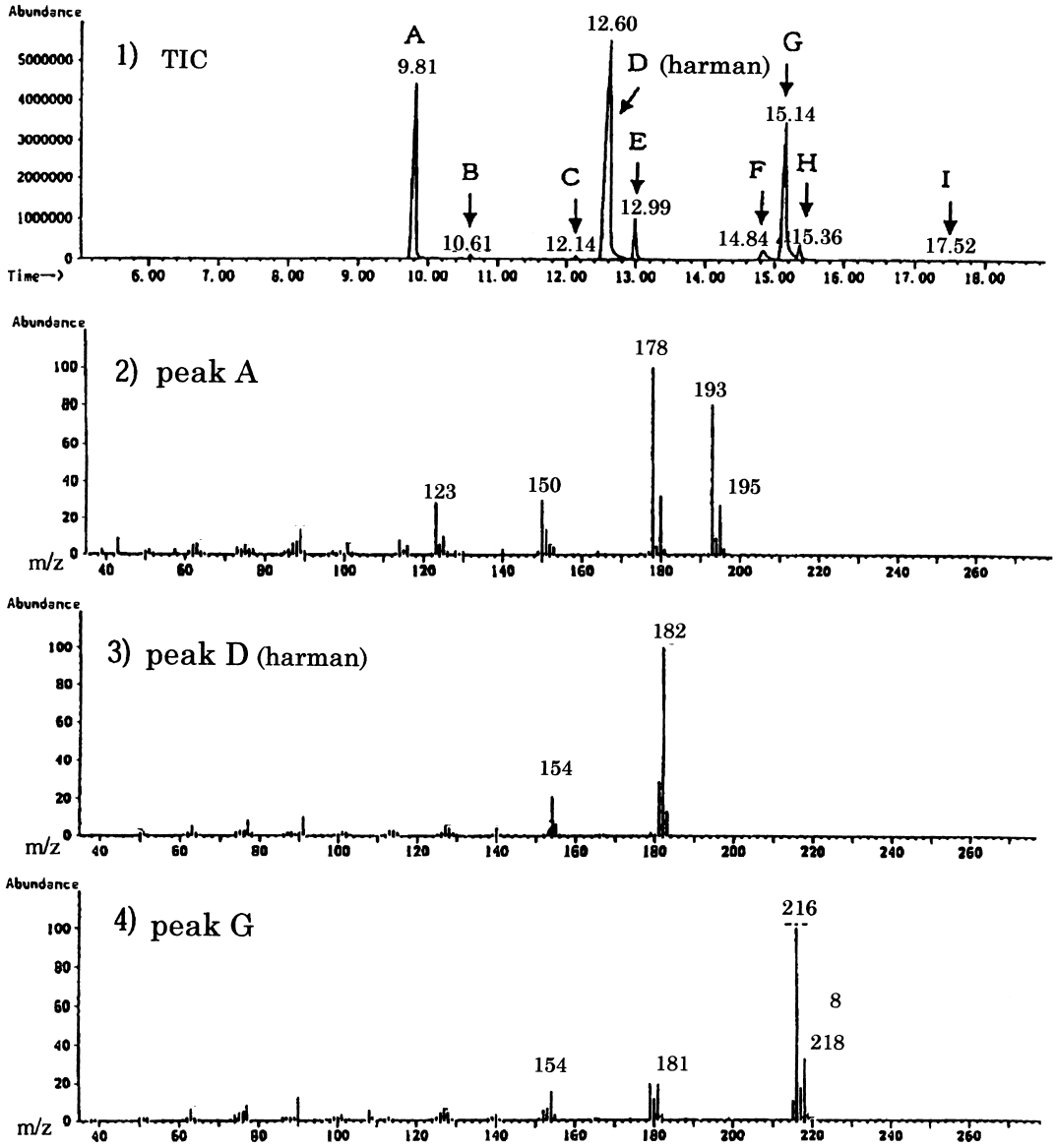

Fig. 2. Total ion chromatogram (TIC) and mass spectra: (1) TIC of the products formed by the reaction of harman with sodium hypochlorite in aqueous medium. Mass spectra of (2) peak A, (3) peak D, (4) peak G.

ranged from 110 to 730 ng/g BR, and mono-chlorinated harmans were identified and found in concentration ranging from 1 to 45 ng/g BR when harman was found at a relatively high concentration.

The ratios of mono-chlorinated harmans to harman, which were calculated from the amounts adsorbed by blue rayon, were 3.7–23.7% for 3-chloroharman, 1.1–5.1% for 6-chloroharman and 0.5–0.7% for 8-chloroharman. Based on the quantification of harman (27–75 ng/l) by extraction with a SDB-XD disk, the amount of chlorinated harman was estimated to be on the order of a few ng/l. This value is a little less than that of mutagenic and carcinogenic 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2)

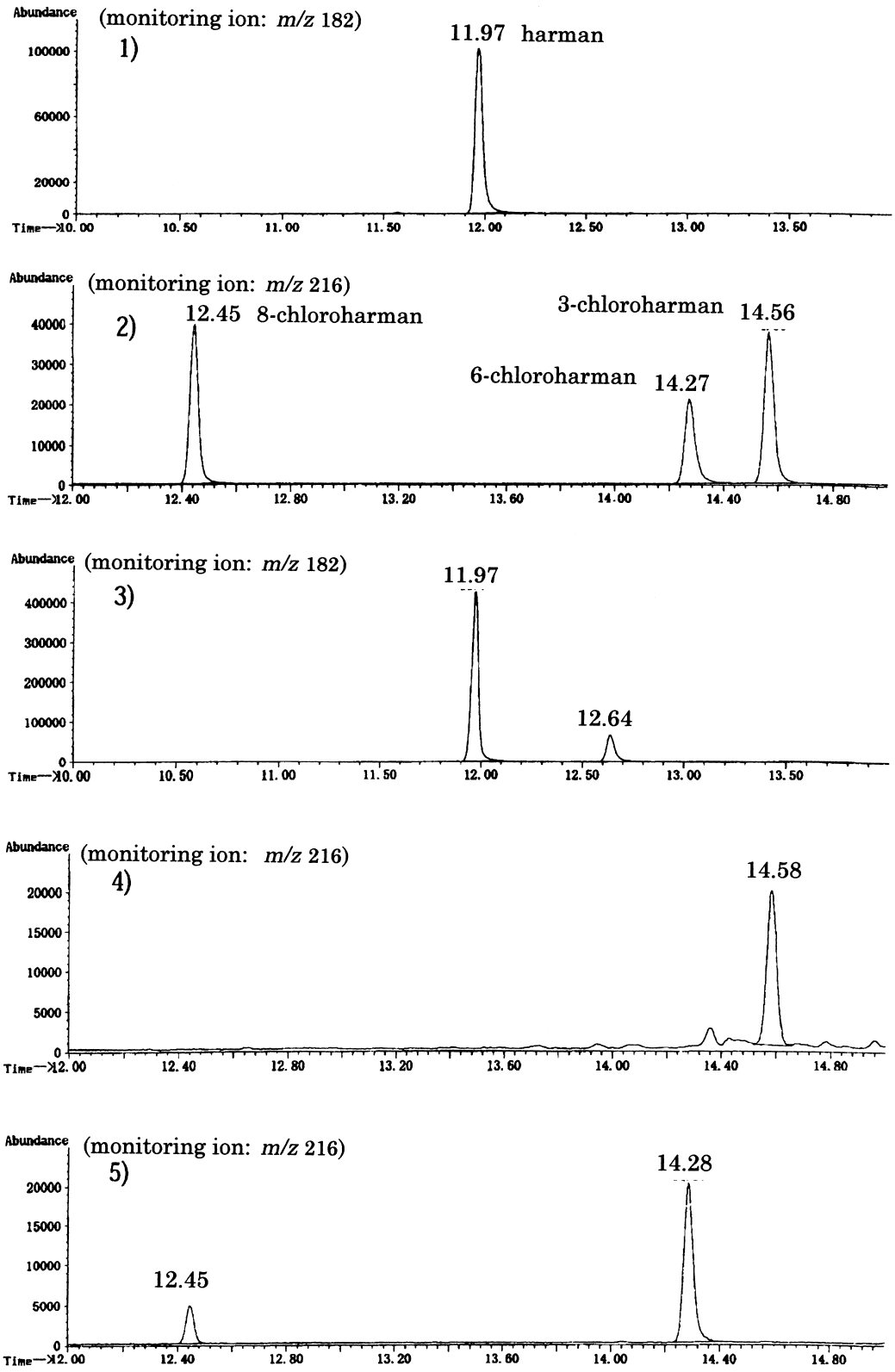

Fig. 3. SIM chromatograms of the compounds absorbed with blue rayon in final effluent from a sewage treatment plant: (1) and (2) authentic compounds, (3) harman in the acetone-solution eluate, (4) 3-chloroharman in the ethyl acetate-solution eluate, (5) 6- and 8-chloroharman in the acetone-solution eluate.

Table 1
Adsorption amounts of harman and the chlorinated derivatives in final effluent from a sewage treatment plant[a]

Date	Harman	6-Chloroharman	8-Chloroharman	3-Chloroharman
21 August	730	37	5	27
4 September	110	Trace[b]	Trace	Trace
2 November	190	2	1	45

[a] ng/g of blue rayon.
[b] Trace: <1 ng/g of blue rayon.

which was detected (on the order of 10 ng/l) in final effluent from a sewage treatment plant [12].

Co-mutagenic mono-chlorinated harmans were detected along with harman in final effluent from a sewage treatment plant. Although their concentrations were not so high, chloroharmans have evidently contaminated river water for a long time. With regard to environmental pollutants, chloroharman should be considered a toxic organohalogen compound, since it seems likely that the chlorinated derivative exhibits toxicity other than co-mutagenicity. In any case, the occurrence of chloroharmans is likely due to disinfection with sodium hypochlorite in sewage treatment plants.

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