Systematic analysis and the overall toxicity evaluation of dioxins and hexachlorobenzene in human milk

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Introduction

The hexachlorobenzene (HCB), a type of organochlorine pesticide (OCP), was used as a fungicide for seed, and as a wood preservative. Also, HCB exists in the by-products found in the manufacturing process of chlorinated organic chemicals, and is generated by garbage incineration[1]. The HCB is a so-called, unintended toxic pollutant as well as dioxins, and HCB is then specified for Persistent Organic Pollutants (POPs). According to a recent study, it was pointed out that HCB binds to the aryl hydrocarbon (Ah) receptor[2,3], resulting in dioxin-like effects and bioaccumulates. Therefore, the overall toxicity evaluation of dioxins and HCB in human body, especially in human milk, should be examined, because HCB is universally detected in human milk. Until now, many studies regarding the dioxins or OCPs polluted in human milk have been reported. However, there are only a few reports that analyze both dioxins and HCB in the same sample[4], because repeated sampling and large amounts of samples of human milk were generally difficult to acquire. Moreover, few studies are available for the overall toxicity evaluation of dioxins and HCB in human milk.

The aim of the present study was to develop the systematic analysis method of dioxins and HCB, and to obtain additional information about the overall toxicity evaluation of dioxins and HCB in human milk. The correlation between the HCB residue level and each dioxin isomer in the human milk was also considered.

Materials and Methods

Chemicals: All of the dioxin standards were from Wellington Laboratories. The OCPs were HCB, α-hexachlorocyclohexane (HCH), β-HCH, γ-HCH, δ-HCH, *o,p'*- DDT, *p,p'*-DDT, *o,p'*- DDD, *p,p'*-DDD, *o,p'*- DDE, *p,p'*-DDE, heptachlor and heptachlor epoxide, all of which were from Wako Pure Chemical Industries (Osaka, Japan). Most of the organic solvents such as hexane, acetone, dichloromethane (DCM), toluene, diethylether and ethanol were of dioxin analysis quality from Kanto Kagaku (Tokyo, Japan) or Wako Pure Chemical Industries. All the other chemicals were from PCB analysis quality grade or special quality grade.

Analysis of dioxins and HCB: Human milk samples were collected from 100 Japanese primiparae. Approximately 50 g milk samples were used for the analysis. The sample pretreatment for dioxin analysis was carried out in accordance with the manual compiled by the Ministry of Health, Labour and Welfare, Japan. Briefly, a stable isotope of each congener of the PCDD/Fs and Co-PCBs were added as a surrogate after the fat was extracted from the human milk. The fat was subjected to a concentrated sulfuric acid washing and then to chromatographies such as silica-gel column (1.5g of silica-gel; eluted with 120 mL hexane, followed by 60 mL of 10% DCM/hexane), alumina column (6.5 g of basic alumina; eluted with 60mL of 2% DCM/hexane, followed by 100 mL of 60% DCM/hexane) and activated carbon silica-gel column (0.5 g of activated carbon silica-gel; eluted with 60 mL of 25% DCM/hexane, followed by 100 mL of toluene) as the cleanup operation, followed by the GC/MS measurement for dioxins.

As for the analysis of HCB, the fraction of 2% DCM/hexane eluate from alumina column was evaporated near dryness in vacuo, and the residue was dissolved with 1mL of hexane, followed by the GC-ECD (electron capture detection) measurement. For other OCPs such as heptachlor epoxide and a part of β-HCH, the fraction of the 10% DCM/hexane eluate from the silica-gel column was used in a similar manner. For the remaining β-HCH, the fraction of 25% DCM/hexane eluate from activated carbon silica-gel column was also used in a similar manner.

GC/MS measurement: The PCDD/Fs were analyzed by HR-GC/MS using a JEOL JMS-700 mass spectrometer equipped with a capillary DB-17HT column (30 m x 0.25 mm i.d., film thickness 0.15 μm) in the splitless injection mode (1 μl). The GC program was as follows: 150 °C (1 min) to 220 °C (0 min) at 20 °C/min and subsequently at 4 °C /min to 280 °C, then maintained for 16.5 min at 280 °C. The MS was operated in the selected ion monitoring mode with a mass resolution of 10,000, and the electron impact ionization energy was 38 eV with an ion source temperature of 260°C. The toxic equivalent quantity (TEQ) was calculated using the international toxic equivalency factors (I-TEF, 1988), WHO/IPCS-TEF (1993) or WHO-TEF (1998).

GC-ECD measurement: The HCB and other OCPs were analyzed by GC-ECD using a HP5890 SERIES II (Agilent) equipped with a capillary DB-5.625 column (30 m x 0.25 mm i.d., film thickness 0.25 μm) in the splitless injection mode (1 μl). The GC program was as follows: 70 °C (1 min) to 150 °C (0 min) at 20 °C/min and subsequently at 3 °C /min to 270 °C, then maintained for 10 min at 270 °C. The injector temperature was 200°C and the detector temperature was held at 300°C. The quantification of the OCPs was carried out using the absolute standard curve method.

Results and Discussion

Behavior of HCB and other OCPs in preprocessing process of dioxin analysis: The behavior of the HCB and other OCPs in each column chromatography was examined. In the silica-gel column, the HCB and most of OCPs were eluted in the first fraction (hexane 120mL) except heptachlor epoxide, δ-HCH and a part of β-HCH. The heptachlor epoxide and δ-HCH besides β-HCH of the remainder were eluted in the second fraction (10% DCM/hexane 60mL). In the alumina column, HCB, *o,p'*-DDE and *p,p'*-DDE were eluted in the first fraction (2%DCM/hexane, 60mL). However, the other OCPs excluding β-HCH were eluted neither in the first fraction nor in the second fraction (60% DCM/hexane 100mL). On the other hand, in the activated carbon silica-gel column, all the OCPs were eluted in 25%DCM/hexane 60mL. From the above-mentioned results, the fractionation

of HCB and other OCPs was shown in the flowchart of Fig.1. The HCB and some pesticides such as *o,p'*-DDE, *p,p'*-DDE, heptachlor epoxide, β-HCH and δ-HCH were found to have the possibility to construct a systematic analysis with dioxins using the preprocessing of dioxin analysis. In the present study, HCB, heptachlor epoxide and β-HCH were determined.

Recovery study: The bovine milk samples fortified at a level of 10 ng/g each of HCB, heptachlor epoxide and β-HCH were used for the recovery study. The overall mean recoveries were 60.3 – 70.5 % and the standard deviations (SD) were less than 9%. The β-HCH was calculated by the summation of the recovery from a silica-gel column and an activated carbon silica-gel column as shown in Fig.1.

Investigation of HCB pollution level in human milk: In Table 1, there are summarized levels of OCPs determined in our study concerned with the examination of the set of 100 human milk samples. The residual level of HCB was 4.1 – 91.8 ng/g fat (mean; 33.9 ng/g fat). The heptachlor epoxide and β-HCH were also found in all of the samples. These data suggested that the human milk had been polluted by these persistent organochlorine contaminants.

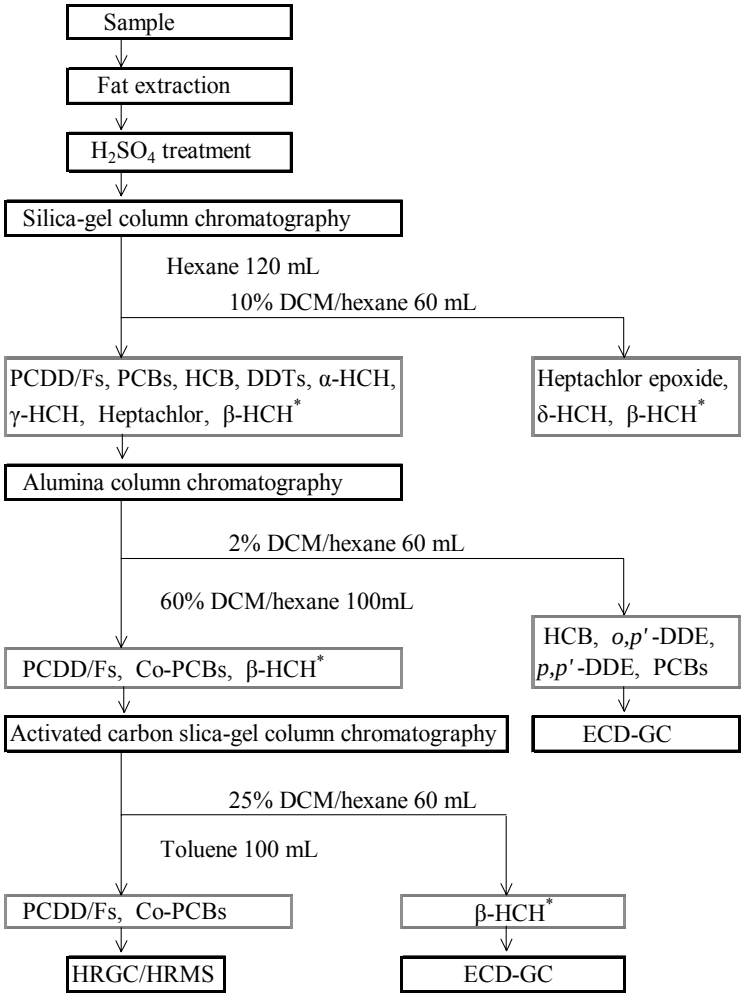

Fig. 1. Flowchart of systematic analysis of dioxins and hexachlorobenzene in human milk

Table 1. Residual concentration of HCB, heptachlor epoxide and β-HCH

Peticide	Mean	Min	Max	SD
	(ng/g fat; n=100)			
HCB	33.9	4.1	91.8	16.2
Heptachlor epoxide	7.4	1.4	22.1	4.0
β-HCH	62.7	8.1	610.3	80.8

Correlation analysis: The Pearson's correlation coefficients among the residue levels of OCPs and each isomer of the dioxin in human milk were examined with the data of 100 samples. The HCB showed a significant positive correlation ($p < 0.01$) with most of the dioxin isomers. On the other hand, heptachlor epoxide and β-HCH showed a poor correlation with the dioxin isomers. In addition, more significant positive correlations were found for the HCB and the dioxin isomers with a high TEF such as 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD and 2,3,4,7,8-PeCDF. These results suggest that the behavior of the exposure root and the cumulative exposure to the human body throughout the lifetime by HCB was assumed to be similar to those of the dioxins.

Overall toxicity evaluation of dioxins and HCB: According to a recent study[3] the binding activity to the Ah receptor of HCB, it is said that the toxicity equivalency factor (TEF) of HCB corresponds to 0.0001. This value is as low as OCDD and OCDF. However, so far it is reported that the contaminated level of HCB is higher than that of the dioxins in human milk. When the HCB toxicity was calculated using the TEF (0.0001), the TEQ of HCB in human milk yielded 0.41-9.2pg TEQ/g fat (Mean value:3.4 pg TEQ/g fat, n=100) (Table 2). It yielded an increase of about 16% (average value) when these results were summed with the TEQ (calculated using I-TEF) of dioxins. Because the mono-ortho-PCBs were not determined in the dioxin analysis at this time, the total TEQ in the WHO-TEF(1998) was calculated using the guessing value (it was assumed that the TEQ mono-ortho-PCBs accounted for about 13% of total TEQ by WHO-TEF). As a result, the increase in TEQ by HCB became about 12%.These data were almost in the same range as those reported in the literature[3], which described that HCB could add 10 - 60 % total TEQ in human milk samples. On the basis of these results, it was understood that evaluating the overall toxicity by adding HCB was necessary for the dioxin toxicity evaluation in human milk.

Table 2. Increase of dioxin toxicity (TEQ) in human milk when including HCB toxicity

	Toxicity (pg TEQ/g fat)*	Increasing rate
HCB	3.4	
Dioxins (I-TEF)	21.8	
Dioxins (WHO-TEF 1998)**	28.5	
HCB + Dioxins (I-TEF)	25.2	116%
HCB + Dioxins (WHO-TEF 1998)	31.9	112%

* Average of 100 human milk samples

** The data of mono-ortho PCBs was calculated using presumed value (13% of Total TEQ).

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