Enantiomeric analysis of 2,2',3,4,4',5',6-heptachlorobiphenyl in the blood of Yusho patients

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Introduction

Yusho is the name given to a food poisoning incident that occurred in 1968 in western Japan after more than 1800 people ingested rice bran oil contaminated with polychlorinated biphenyls (PCBs)[1]. Yusho patients were treated medically immediately after the outbreak. Shortly after the incident, the concentrations of PCBs in the blood of typical Yusho patients were higher than those in a normal control group[2].

Among the 209 PCB congeners, 19 contain a chiral axis; due to the presence of three or four ortho chlorine substituents, these 19 congeners exist as two stable atropisomers that are nonsuperimposable mirror images of each other (chiral PCBs)[3,4]. The PCB manufacturing process results in the formation of racemic mixtures, and physico-chemical processes, such as diffusion, partition, or evaporation, do not result in an atropisomeric enrichment of chiral PCBs[5,6]. On the other hand, biological processes such as biotransformation and protein binding can result in an atropisomeric enrichment of chiral PCBs[7,8]. Thus, the quantification of the enantiomer fraction value (*EF*) of a chiral PCB can be used to assess its biological processes[9].

In this study, we analyzed 2,2',3,4,4',5',6-heptachlorobiphenyl (PCB183, Fig. 1) in the blood of Yusho patients enantioselectively and evaluated the enantiomer-specific accumulation of it.

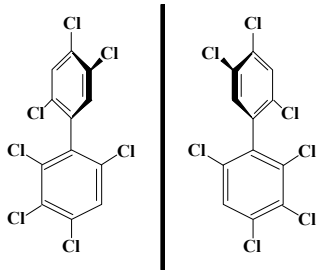

Fig. 1 Enantiomers of atropisomeric 2,2',3,4,4',5',6-heptachlorobiphenyl (PCB183).

Materials and methods

Materials

The blood samples examined in this study were collected twice from 5 Yusho patients (A-E) during medical check-ups several years after the incident (from 1974 to 1976) and then closer to the present (from 2015 to 2016). All 5 of these patients gave informed consent to participate in this study.

Reagents

Acetone, hexane, dichloromethane, toluene, nonane, potassium hydroxide, and anhydrous sodium sulfate were purchased from Kanto Chemical Industries (Tokyo, Japan). Ethanol, distilled water, 10% (w/w) silver nitrate impregnated silica gel, and sulfuric acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). Activated carbon was purchased from Nacalai Tesque (Kyoto, Japan). Sep-Pak Florisil 3 cc Vac RC Cartridges were purchased from Waters (Milford, MA, USA). Native and $^{13}C_{12}$-labeled PCBs were purchased from Wellington Laboratories (Guelph, Ontario, Canada).

Analysis of chiral PCB183

Chiral PCB183 analysis was performed using an Agilent 7890A (Agilent Technologies, Santa Clara, CA, USA) gas chromatograph with a 7693A series auto injector interfaced with a JMS-800D UltraFOCUS™ (JEOL, Tokyo, Japan) high-resolution gas chromatograph–high-resolution mass spectrometer (HRGC/HRMS). PCB183(+) and PCB183(-) were separated on a BGB172 (30 m × 0.25 mm i.d., 0.18 µm film thickness, BGB Analytik, Adliswil, Switzerland). The HRGC/HRMS conditions are shown in Table 1.

Table 1 High-resolution gas chromatograph-high resolution mass spectrometry conditions

GC (7890A, Agilent Technologies)	
Column	BGB172, 30 m × 0.25 mm i.d, film thickness: 0.18 μm
Oven temp.	Initial temperature at 120 °C hold for 2 min → heat up to 250 °C at 2 °C/min → hold at 250 °C for 23 min
Injection port temp.	230 °C
Injection mode and volume	Splitless (purge time, 1 min), 1 μL
Carrier gas	Helium with a purity of greater than 99.999% 1.0 mL/min constant flow
MS (JMS-800D UltraFOCUS^{TM}, JEOL)	
Resolution	Equal to or greater than > 10000 (10% valley)
Ionization voltage	38 eV
Ionization current	500 μA
Ion source temperature	260 °C
Accelerating voltage	10 kV

Blood sample preparation

The blood samples were prepared according to reported methods[10,11].

(1) Blood samples of Yusho patients collected from 1974 to 1976

The blood samples of Yusho patients collected from 1974 to 1976 were stored in a medical freezer for over 40 years. All of the samples were in a dry state and deposited on the inner walls (the inside sidewalls and the bottom) of sealed glass containers. Two-hundred picograms of internal standard was added to each sample, followed by 6 mL of 1.5 mol/L potassium hydroxide/ethanol solution and 4 mL of distilled water. After 3 mL of hexane was added, the mixture was shaken strongly for 10 sec and then centrifuged at 3000 rpm for 10 min to recover the hexane layer. This extraction was repeated three times, and the extract of each sample was added to a Sep-Pak Florisil 3 cc Vac RC Cartridge. Each cartridge was eluted with 8 mL of hexane, and the eluate was collected. Nonane (50 μL) was added to the eluate, and solvent was evaporated from the mixture at 40 °C, followed by reduction to 50 μL under a stream of nitrogen gas. Finally, 200 pg of syringe spike was added to prepare the HRGC/HRMS sample.

(2) Blood samples of Yusho patients collected from 2015 to 2016

The extraction was performed with an accelerated solvent extractor (ASE) system. After 14 hours of freeze-drying, 200 pg of internal standard was added, and lipid was extracted from each sample. The following parameters were used for this extraction: a pressure of 2000 psi and a temperature of 150 °C, a static time of 10 minutes, and acetone: hexane (1: 3, v/v) as the extraction solvent. This extraction was repeated twice, and the extract was concentrated near dryness. The lipid was dissolved in 5 mL of hexane and treated with 3 mL of sulfuric acid, then centrifuged at 3000 rpm for 10 min to recover the hexane layer. The hexane layer was applied to a 10% (w/w) silver nitrate impregnated silica gel column (0.5 g). The column was eluted with 15 mL of hexane, and the eluate was applied to a 0.1% (w/w) activated carbon sodium sulfate column (0.5 g). The column was eluted with 10 mL of dichloromethane: hexane (1: 9, v/v) and 25 mL of toluene. Nonane (50 μL) was added to the eluate, and solvent was evaporated from the mixture at 40 °C, followed by reduction to 50 μL under a stream of nitrogen gas. Finally, 200 pg of syringe spike was added to prepare the HRGC/HRMS sample.

Calculation of enantiomer fraction value

The *EF* value was calculated based on the peak areas of PCB183(+) and PCB183(-) to quantify the enantiomer-specific accumulation of PCB183.

$$EF = \frac{Peak\ area\ of\ PCB183(+)}{Peak\ area\ of\ PCB183(+)\ and\ Peak\ area\ of\ PCB183(-)} \quad (1)$$

An *EF* value can range from 0 to 1, with 0.5 representing a racemic mixture.

Statistical analysis

The *EF* values of the blood samples of Yusho patients collected from 1974 to 1976 and those from 2015 to 2016 were compared. The Shapiro-Wilk test was used to determine data distribution, and the paired *t*-test was applied to compare the *EF* values from group to group. Statistical significance was considered when $p < 0.05$. All statistical analyses were performed using the statistical software R version 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria).

Results and discussion

Calculation of enantiomer fraction value

In this study, we determined chiral PCB183 in the blood of Yusho patients. As shown in Fig. 2, we separated PCB183(+) and PCB183(-). When the *EF* value was calculated with Eq. 1, that of the standard was close to racemic (mean 0.497, $n = 8$). On the other hand, the *EF* values of the blood samples of the Yusho patients were

0.573-0.750 (Table 2), and atropisomeric enrichment of chiral PCB183 was observed. A similar result was obtained from human breast milk[12].

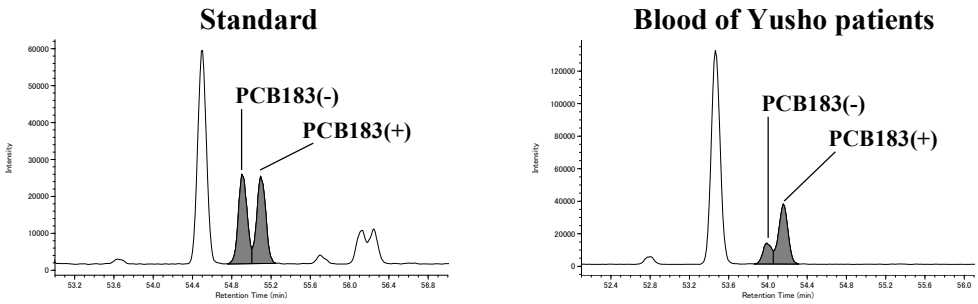

Fig. 2 Chromatogram of enantioselective analysis of 2,2',3,4,4',5',6-heptachlorobiphenyl (PCB183).

Table 2 Enantiomer fraction values (*EF*) of the blood of Yusho patients

Patient	Sampling date (year)	
	1974-1976	2015-2016
A	0.657	0.750
B	0.573	0.739
C	0.644	0.703
D	0.637	0.749
E	0.623	0.719

Comparison of enantiomer fraction values by sampling period

The *EF* values of the blood samples of Yusho patients collected from 1974 to 1976 were 0.573-0.657 (mean 0.627), and those of the 2015 to 2016 samples were 0.703-0.750 (mean 0.732) (Fig. 3). The *EF* values were proved, by the Shapiro-Wilk test, to be distributed normally within the samples of Yusho patients collected from 1974 to 1976 ($W = 0.898$, $p = 0.302$) and those from 2015 to 2016 ($W = 0.892$, $p = 0.368$). Paired *t*-test analysis showed that the *EF* values of the blood samples of Yusho patients collected from 1974 to 1976 were significantly lower than those of the 2015 to 2016 samples ($t = -5.98$, $p = 0.00392$). This fact indicates that PCB183(+) has greater accumulation potential than PCB183(-) in the human body. We might be able to distinguish between Yusho patients and the normal control group by quantifying the *EF* value of chiral PCB183.

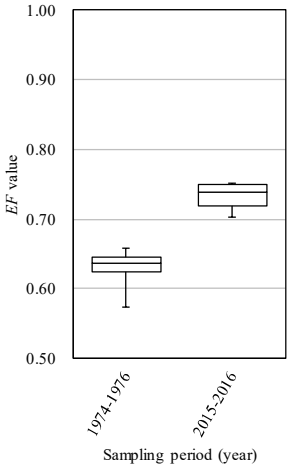

Fig. 3 Comparison of enantiomer fraction (*EF*) values between the blood samples of Yusho patients collected from 1974 to 1976 and from 2015 to 2016.

More work is needed to understand the toxicokinetics of chiral PCB183 and the underlying biological processes. In the future, we will investigate time-dependent changes in EF values of chiral PCB183 in the blood of Yusho patients.

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