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Polychlorinated Quaterphenyl Concentrations in the Blood and Their Patterns in Subjects Examined for a Possible Diagnosis of Yusho from FY 2009 to 2019

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Abstract

Since 1985, blood concentrations of polychlorinated quaterphenyls (PCQs), which are congeners that accumulate in the bodies of typical Yusho patients in the same way as PCBs, have been monitored by the Study Group for Yusho. We present here the tendencies and characteristics of blood PCQ concentrations in subjects examined for a possible diagnosis of Yusho from FY 2009 to 2019. The peak patterns of PCBs in the blood tended to be close to those of a typical Yusho patient as the blood PCQ concentration increased. The result of the secular change of PCQ concentration in a typical Yusho patient, suggested that lipophilic PCQ congeners were hardly excreted even in high-exposure cases. Almost all of the blood PCQ concentrations in subjects born after the rice oil poisoning incident were as with those of healthy individuals. Therefore we concluded that PCQ congeners had hardly transferred from Yusho patients to the next generation. The usefulness of blood PCQ concentration as a diagnostic criterion for Yusho is still unchanged, and a continuous follow-up survey will be important in the future.

Key words : Polychlorinated Quaterphenyl (PCQ), Blood, Yusho

Introduction

A mass food poisoning occurred in western Japan in 1968. The incident is called Yusho, or oil disease, as it was caused by the ingestion of rice oil contaminated with polychlorinated biphenyls (PCBs). Polychlorinated quaterphenyls (PCQs) are among the typical chemical substances contained in high concentrations in the patients poisoned in the incident, and these are believed to have been unintentionally generated when PCBs were used as a heating medium[1]. PCQs are rarely detected at trace level in the blood of healthy individuals[2]. On the other hand, PCQs were specifically detected in the blood of Yusho patients in 1981[3]. For this reason, a Yusho Study group, created by The Ministry of Health, Labor and Welfare of Japan, added PCQ concentrations in the blood to the diagnostic criteria for Yusho in 1981, and the concentrations have served as characteristic and useful information on Yusho. The criteria for blood PCQ concentrations in Yusho diagnoses are an "Abnormally high concentration" of 0.1 ng/g or more and a "Normal concentration" of 0.02 ng/g or less ; 0.03 to 0.09 ng/g between the two is regarded as the "Boundary region concentration".

According to previous reports[4)~6)] that analyzed the tendencies and characteristics of blood PCQ concentrations in subjects examined for a

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possible diagnosis of Yusho in Fukuoka Prefecture from FY 1999 to 2008, the blood PCQ concentrations of the typical Yusho patient decreased more gradually from the start of measurement. Therefore, it was obvious that PCQ congeners were gradually excreted from the body, but the degree of excretion decreased year by year. Moreover, it was considered a future task to verify whether or not PCQs were transferred to the generation born after 1989 from the viewpoint of the health effect on children, while PCQs were detected in the blood of subjects born before the 1968 incident[6].

The PCQ analytical method in blood was first developed by Maeda et al.[7]. This method consists of both the chemical derivatization of PCQs to octadecachloro quaterphenyls (ODCQs), which are completely chlorinated forms, and the determination of ODCQs using gas chromatography with an electron capture detector (GC-ECD). After that, the PCQ analytical method in the blood was improved, achieving higher sensitivity[8], higher speed[4], and greater efficiency[9]. Currently, each institution conducts PCQ inspections according to the improved analytical method. In addition, the quality control of analytical methods has been required recently to ensure the reliability of inspection results.

In this study, we attempted to investigate the tendencies and characteristics of blood PCQ concentrations in subjects examined for a possible diagnosis of Yusho from FY 2009 to 2019. First, to control the quality of the analytical method, the method's accuracy was confirmed using a positive control sample of PCQs. We then analyzed the relationship between the blood PCQ concentrations of the subjects and their peak patterns of PCBs (PCB patterns), which were closely related to the severity of clinical symptoms of Yusho patients[10], as well as the secular change of the blood PCQ concentrations in a typical Yusho patient. Finally, we reported the results of our investigation of the blood PCQ concentrations of subjects born after 1968.

Materials and Methods

1. Materials

The following were obtained from Wako Pure Chemical Industries : potassium hydroxide for a guaranteed reagent, 30% fuming sulfuric acid for Wako 1st grade, sulfuric acid for analysis of poisonous metals, 20% hydrochloric acid for a super special grade, (+) -sodium tartrate dihydrate for a guaranteed reagent, sodium hydrogen carbonate for a guaranteed reagent, chloroform for residual pesticide/PCB analysis, and n-nonane for analysis of dioxins. Ethanol and n-hexane were used for analysis of dioxins (Kanto Chemical). Distilled water with a n-hexane wash was residual pesticide analysis grade (Kanto Chemical). Anhydrous sodium sulfate, diethyl ether, and dichloromethane, manufactured by Kanto Chemical, were used for the residual pesticide/PCB analysis. Antimony (V) chloride was PCB analysis grade (Sigma-Aldrich).

Florisil (particle size : 150-250 μm) for column chromatography (made by Kanto Chemical) was previously activated at 130℃ for 3 hours and used. Alumina 90, Activated, Neutral (70-230 mesh) for column chromatography (made by Merck Millipore) was previously added to distilled water to 2% after activation at 130℃ for 3 hours and used.

2. Equipment

Electric analytical scale : AG285 (Mettler Toledo)

Oil bath : EO-200RD (AS ONE)

Electric drying oven : DRN420DB (ADVANTEC)

Centrifuge : 2410 (Kubota)

GC-ECD : 7890B (^{63}Ni-ECD)(Agilent Technologies)

3. Samples

PCQs were analyzed in a total of 1838 blood samples collected from subjects examined for a possible diagnosis of Yusho across Japan by the Study Group of Yusho from FY 2009 to 2019. In

2011 we prepared the positive control sample used for quality control, and the stocked sample was distributed to the other institution before the start of annual PCQ inspections. Since the positive control was reprepared in 2018, the new lot was used in from 2018 on.

4. Preparation of test solution

The test solution was prepared according to a previous report[4)9)]. Approximately 2 g of a well-mixed blood sample was accurately weighed in a 10 mL round-bottom test tube. After adding 3.5 mL of 1.5 N potassium hydroxide/ethanol and mixing well, the mixture was heated in a water bath at 80℃ for 1 hour. After cooling to room temperature, 2 mL of n-hexane was added and extracted by shaking. The extract was centrifuged (3,000 rpm, 1 min) and the n-hexane layer was collected in another test tube. This extraction was performed a total of three times. The recovered n-hexane layer was washed with 2 mL of distilled water and centrifuged (3,000 rpm, 1 min). The n-hexane layer was dehydrated by passing it through a Pasteur pipette filled with anhydrous sodium sulfate (1.5 g), eluted with 2 mL of n-hexane, and combined with the above solution. This solution was dried under a nitrogen stream and redissolved in 1 mL of n-hexane, loaded onto a Pasteur pipette filled with anhydrous sodium sulfate (0.2 g)/florisil (0.5 g), and eluted with 7 mL of 5 % diethyl ether/n-hexane. The eluate was dried under a nitrogen stream and dissolved in 2 mL of n-hexane. This solution was added to 1 mL of 10% fuming sulfuric acid, and the mixture was stirred well and centrifuged (3,000 rpm, 1 min), after which the sulfuric acid layer was discarded. This operation was repeated until the sulfuric acid layer became transparent. The n-hexane layer was recovered and concentrated to dryness, and then the solvent was completely distilled off with a vacuum desiccator. After 0.5 mL of antimony(V) chloride was added and the tube was sealed, it was heated at 200℃ for 3 hours to completely chlorinate. While the tube cooled in an ice bath, 2 mL of chloroform was added and stirred well, and then 0.5 mL of 20% hydrochloric acid was added and mixed. Further, 2 mL of 20% hydrochloric acid was added, the mixture was stirred and centrifuged (3,000 rpm, 1 min), and then the hydrochloric acid layer was discarded. This operation was performed a total of three times. Subsequently, the chloroform layer was washed in the order of 2 mL of distilled water, 2 mL of a 5% aqueous (+)-tartaric acid solution, 2 mL of 5% aqueous sodium hydrogen carbonate, and 2 mL of distilled water. The chloroform layer was passed through a Pasteur pipette filled with anhydrous sodium sulfate (1.5 g), eluted with 6 mL of n-hexane, and combined with the above solution. This solution was dried under a nitrogen stream, redissolved in 1 mL of n-hexane, loaded onto a Pasteur pipette filled with anhydrous sodium sulfate (0.2 g)/alumina (0.25 g), and eluted with 6 mL of 2% dichloromethane/n-hexane. Finally, the eluate was dried under a nitrogen stream and then dissolved in an appropriate amount of n-nonane (usually 0.2 mL) to prepare a test solution.

5. GC-ECD conditions

Capillary column : Quadrex 007-65HT (0.25 mm I.D.× 25 m, film thickness 0.1 μm) ; temperature program : 80℃, hold for 1 min and 30℃/min to 320℃, hold for 30 min ; inlet temperature : 300℃ ; injection method : Pulsed splitless ; injection volume : 4 μL ; Carrier gas : Helium ; Detector temperature : 350℃ ; Makeup gas : nitrogen.

6. Quantification of PCQ by absolute calibration curve method

Five nanograms of PCQ standard was taken and the operations were performed after complete chlorination, as described in section 4. A standard solution for the calibration curve of ODCQs corresponding to a PCQ concentration of 2 ng/mL was prepared by dissolving in 2.5 mL of n-nonane. The ODCQ standard solution (corresponding to 2 ng/mL as PCQs) was appropriately

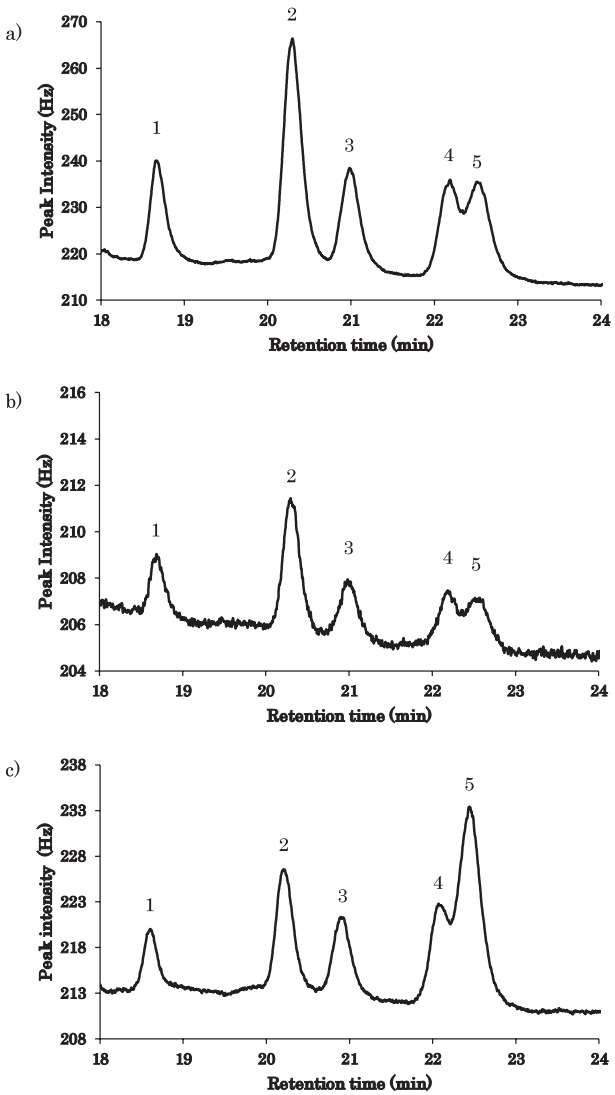

Fig. 1 The gas chromatograms of six skeletal types of perchlorinated PCQs (ODCQs)
Peak 1 : 2,2'-ODCQ, Peak 2: 2,3'-ODCQ, Peak 3 : 2,4'-ODCQ, Peak 4 : 4,4'-ODCQ + 3,3'- ODCQ, Peak 5 : 3,4'-ODCQ
a) ODCQ standard (2 ng/mL as PCQs)
b) ODCQ standard (0.2 ng/mL as PCQs)
c) Sample blood of a typical Yusho patient

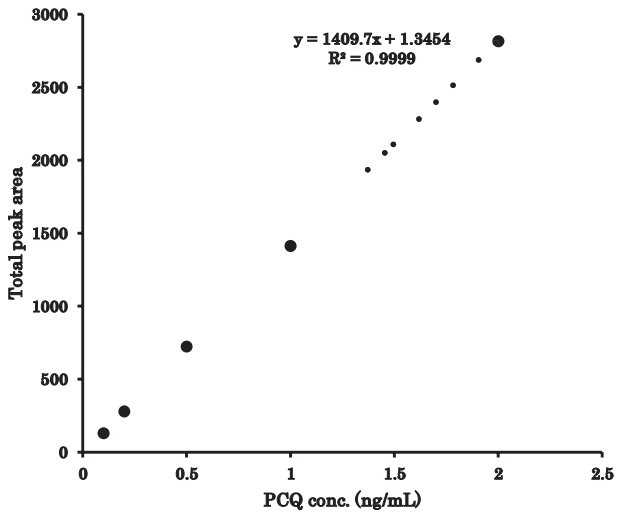

Fig. 2 PCQ calibration curve
The concentration range was 0.1-2.0 ng/mL (five points). The correlation coefficient (R^2) was 0.9999 in this range, and good linearity was obtained.

diluted with n-nonane to prepare ODCQ standard solutions (0.1, 0.2, 0.5, and 1 ng/mL as PCQs) for the calibration curve. Each standard solution was measured under the analysis conditions detailed in section 5, and a calibration curve was created using the total area of the five peaks obtained from each standard solution. The concentration of PCQs was calculated by interpolating the total of the peak areas obtained by measuring the test solution prepared according to section 4 into the created calibration curve.

7. Quality control

The positive control was analyzed in parallel with the PCQ inspection by two institutions (A and B) from FY 2011 to 2019.

Results and discussion

1. Standard curve

Typical gas chromatograms of ODCQ isomers obtained by completely chlorinating the PCQ standard are shown in Fig. 1 : (a) maximum concentration on the calibration curve (2 ng/mL as PCQs) and (b) concentration corresponding to the lower limit of quantification (0.02 ng/g) (0.2 ng/mL as PCQs). As shown in Fig. 1, five peaks were obtained from six ODCQ isomers. The created calibration curve is shown in Fig. 2. The correlation coefficient (R^2) of this calibration curve was 0.9999 in the range of 0.1-2.0 ng/mL as PCQs, and good linearity was obtained.

2. Quality control

To confirm the accuracy of the PCQ analytical method, the positive control was analyzed in

Table 1 Analytical values of positive control in FY 2011 to 2019 inspections at each institution

Lot 1

Institution	PCQ conc. (ng/g)							Average (ng/g)	SD*1 (ng/g)	%RSD*2
	2011	2012	2013	2014	2015	2016	2017			
A	1.6	1.5	1.3	1.8	1.7	1.8	1.8	1.6	0.19	11.6
B	1.6	1.7	1.5	1.5	1.9	1.6	1.5	1.6	0.15	9.1

Lot 2

Institution	PCQ conc. (ng/g)		Average (ng/g)
	2018	2019	
A	1.6	1.3	1.5
B	1.4	1.4	1.4

The positive control was newly prepared in 2018 (Lot 2).

*1 : Standard deviation

*2 : Relative standard deviation ; SD/Average × 100

parallel with the samples from FY 2011 to 2019. As mentioned above, the reprepared positive control was used in FY 2018 and 2019. Table 1 shows the PCQ concentrations in the positive control for each year. The average concentrations in the positive control from FY 2011 to 2017 were as follows : institution A, 1.6 ± 0.19 ng/g (relative standard deviation (RSD), 11.6%) and institution B, 1.6 ± 0.15 ng/g (RSD, 9.1%), which were almost the same. This accuracy reached the target value (RSD < 15%)[11]. The average PCQ concentrations of positive control in FY 2018 and 2019 were in good agreement between the two institutions. On the basis of these results, this analytical method was considered to be robust and to have excellent reproducibility.

3. PCQ measurement results

The blood PCQ concentrations of a total of 1838 subjects were measured from FY 2009 to 2019. Fig. 1 (c) shows an example of a gas chromatogram of a Yusho patient with a high blood PCQ concentration. Five peaks were obtained as in the standard solution.

A total of 323 blood samples from subjects had PCQ above the lower limit of quantification (0.02 ng/g). Table 2 shows the total number of subjects by blood PCQ concentration levels and PCB patterns. Chromatogram patterns of blood PCBs were classified into four types : Type A, typical Yusho pattern ; Type C, pattern commonly observed in healthy individuals ; Types B and BC : intermediate patterns between Types A and C[10]. A total of 20 subjects had a blood PCQ concentration of 1 ng/g or higher (concentration level 4), and all of their PCB patterns were Type A or B. At each PCQ concentration, the ratio of combined, Type A and B patterns to the total were 65% at PCQ concentration level 3, 29% at level 2, and 27% at level 1. The higher the PCQ concentration, the higher the ratio tended to be. When the blood PCQ concentration was below the lower limit of quantification (PCQ concentration level 1), many cases (67%) had the Type C pattern. On the other hand, in some cases PCQs were not detected even when the PCB pattern was Type A or B.

The boxplot in Fig. 3 shows the distribution of blood PCQ concentrations by PCB pattern (Types A, B, BC, and C) for a total of 323 subjects with PCQs above the lower limit of quantification. The average concentrations of PCQs by PCB patterns were as follows : Type A, 1.2 ng/g ; B, 0.33 ng/g ; BC, 0.17 ng/g ; C, 0.08 ng/g. It was found that the higher the average PCQ concentration, the closer to the pattern of a typical Yusho patient. This result was similar to the distribution of PCQ concentrations in Yusho diagnoses in Fukuoka Prefecture in FY 2004 and 2005 reported by

Table 2 Total numbers of subjects classified by PCQ concentration level and PCB pattern in the FY 2009 to 2019 inspection

Conc.Level	PCQ conc. (ng/g)	PCB pattern				No Pattern*	Total
		A	B	BC	C		
1	ND (＜0.02)	153	256	85	1,015	6	1,515
2	0.02≦, ＜0.1	27	29	27	107	0	190
3	0.1≦, ＜1	19	55	15	24	0	113
4	1≦	15	5	0	0	0	20

*PCB was not detected above the lower limit of quantification, so PCB patterns could not be analyzed.

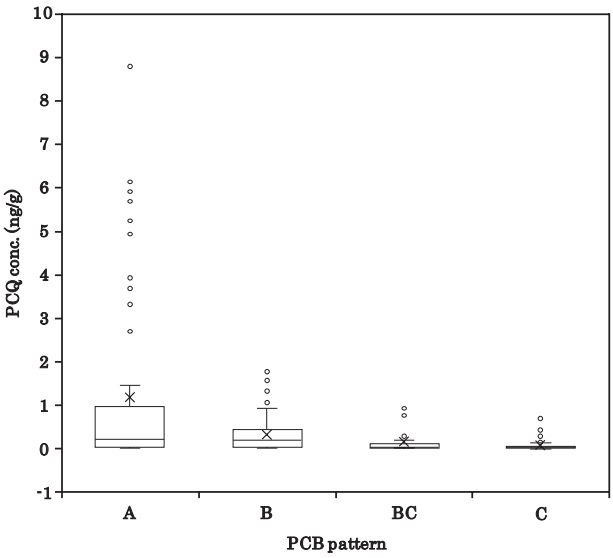

Fig. 3 Boxplot of PCQ concentrations of subjects according to PCB patterns in the FY 2009 to 2019 inspection
The mark "×" means the average value of each pattern.
The bar in the box indicates the median of each pattern.

Ashizuka et al. [4)5)]. The medians of PCQ concentration by PCB patterns were as follows : Type A, 0.22 ng/g ; B, 0.22 ng/g ; BC, 0.06 ng/g ; C, 0.04 ng/g. The concentrations of Types A and B were about four times that of Type BC and six times that of Type C. On the basis of these results, it was considered that the blood PCQ concentrations were related to the PCB patterns and still a characteristic information on Yusho patients.

4. The secular change of PCQ concentrations in a typical Yusho patient

To understand the secular change in the blood PCQ concentrations of Yusho patients, we observed the time trend of PCQ concentrations in the blood of a typical Yusho patient (PCB pattern : Type A), from FY 1986 to 2019 (Fig. 4). The blood PCQ concentrations tended to decrease between FY 1986 and 2004, but did not decrease after FY 2004 and instead fluctuated between 3.9 and 8.8 ng/g. This fluctuation might not be the result of new exposure from the outside or biosynthesis in the body, but rather of PCQs accumulated in the body being transferred to the blood due to weight loss or changes in physical condition resulting from medical issues unrelated to Yusho, causing

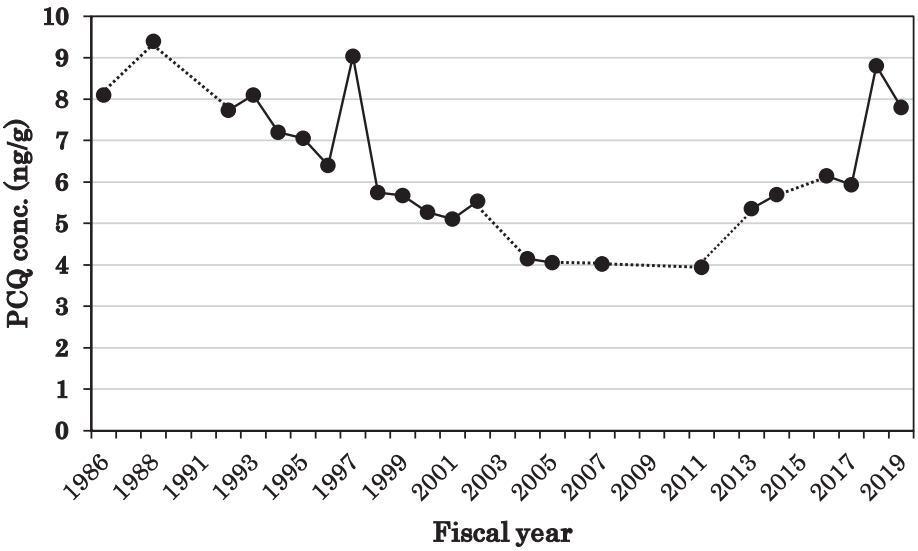

Fig. 4 The secular change of PCQ concentration in a typical Yusho patient (PCB pattern : Type A)
Fiscal years without dots are years when no inspection was conducted.

Table 3 The analytical results of sample bloods obtained from subjects born after 1968 and who showed PCQ concentrations above the lower limit of quantification.

Subject	Year of inspection	Year of birth	PCQ conc. (ng/g)
A	2009	1970	0.04
B	2015	1972	0.02
C	2015	1975	0.02
D	2016	1970	0.02

the apparent blood PCQ concentration to change. Even now, about 50 years after this food poisoning incident, it is presumed that PCQs absorbed in the body due to the large amount of exposure at that time are still present in the blood and remain without being excreted. These results also suggested that lipophilic PCQ congeners were hardly excreted even in high-exposure cases, the same as with a previously reported congener in blood, 2,3,4,7,8-pentachlorodibenzofuran (PeCDF)[12].

5. PCQ concentrations in subjects born after 1968

To verify the presence or absence of the transition of PCQs to the next generation, we investigated the blood PCQ concentrations from FY 2009 to 2019 in subjects born after 1968. A total of 455 blood samples were obtained from subjects born after 1968. Of these, 359 were taken from subjects born between 1969 and 1989, and 96 blood samples were taken from subjects born between 1990 and 2009. Table 3 shows the inspection results of 4 cases in which the blood PCQ concentrations were detected above the lower limit of quantification. The years of birth ranged from 1970 to 1975, and the PCQ concentrations ranged from 0.02 to 0.04 ng/g, which were almost the same as those of healthy individuals. From these results, we concluded that PCQ congeners had hardly transferred from the Yusho patients to the next generation born after 1968.

Finally, the usefulness of blood PCQ concentration as a diagnostic criterion for Yusho is still unchanged, and a continuous follow-up survey will be important in the future.

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（和文抄録）

2009-2019 年度の油症検診における血中のポリ塩化クアテルフェニルの濃度と傾向

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本研究では，厚生労働省油症研究班が実施した 2009 年から 2019 年度の油症検診における受診者の血中ポリ塩化クアテルフェニル（PCQ）濃度の傾向及び特徴を把握することを目的とした．検査に用いた PCQ 分析法の精度は良好な結果であり，本分析法は再現性に優れた頑健な方法であることを確認した．2009 年から 2019 年度の油症検診で採取したのべ 1838 名の血液試料中の PCQ 濃度を解析した結果，血中 PCQ 濃度レベルが高いほど，PCB パターンは油症患者のパターンに近いことが示された．また，1986 年から 2019 年度にかけて，典型的な油症患者の血中 PCQ 濃度の経年傾向を観察した．測定開始から約 14 年間見られた減少傾向は，2004 年度以降見られず，血中 PCQ 濃度は 3.9～8.8 ng/g の間で変動していた．事件発生から約 50 年経過した現在でも，大量の暴露によって体内に吸収された PCQ は血中に存在し，排泄されずに残留していることがわかった．ライスオイルによる食中毒事件発生後（1969 年以降）に出生した検診受診者の PCQ 濃度は，検出しないまたは健常者と変わらない濃度レベルであり，次世代への PCQ の移行はほとんどないと考えられた．血中 PCQ 濃度の油症診断基準としての有用性はいまだに不変であり，今後も継続的な追跡調査が重要である．

キーワード：ポリ塩化クアテルフェニル（PCQ），血液，油症