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DETERMINATION OF POLYCHLORINATED DIBENZOFURANS IN TISSUES OF PATIENTS WITH 'YUSHO'

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Abstract— Tissues of patients with 'Yusho' and of persons not suffering from this disease were analysed for polychlorinated biphenyls (PCBs) and polychlorinated dibenzofurans (PCDFs) by column chromatography, gas chromatography, and gas chromatography–mass spectrometry. Both PCBs and PCDFs were identified in the tissues of patients with Yusho, while only PCBs were detected in the tissues of the other persons studied. In contrast to the PCBs, which were found far more in the adipose tissue than in the liver, PCDFs occurred in very similar concentrations in these two types of tissue. The persisting PCDFs in the tissues of Yusho patients were almost exclusively penta and hexachloro derivatives.

INTRODUCTION

Polychlorinated biphenyls (PCBs) are known to accumulate in the human body as a result of their ingestion in foods, mainly fish. Since polychlorinated dibenzofurans (PCDFs) have been reported to contaminate commercial PCB formulations (Bowes, Mulvihill, DeCamp & Kende, 1975; Bowes, Mulvihill, Simoneit, Burlingame & Risebrough, 1975; Nagayama, Kuratsune & Masuda, 1976; Nagayama, Masuda & Kuratsune, 1975; Roach & Pomerantz, 1974; Vos, Koeman, van der Maas, ten Noever de Brauw & de Vos, 1970) the human body may also be contaminated with PCDFs, which are more toxic than PCBs. PCBs are known to have been the cause of a mass food poisoning which affected more than 1000 persons in western Japan in 1968 (Kuratsune, Yoshimura, Matsuzaka & Yamaguchi, 1972; Tsukamoto *et al.* 1969). The disease is called 'Yusho' i.e. oil disease, because the patients were proved to have consumed a commercial rice oil contaminated with a large amount of Kanechlor-400 (KC-400), a Japanese PCB formulation. Recently, a sample of unused KC-400 and samples of the rice oil consumed by patients with Yusho were found to be contaminated with 20 and 5 ppm PCDF, respectively, indicating that the patients had ingested PCDFs with the PCBs (Nagayama *et al.* 1975 & 1976).

All these findings made it imperative to analyse tissues of unaffected persons and of patients with Yusho for their possible content of PCDFs. This paper describes the main results of such an investigation.

EXPERIMENTAL

Materials analysed. Samples of the liver, mesenteric adipose tissue and a few other organs of three Yusho patients who had died were supplied from the Department of Pathology, School of Medicine, Yamaguchi University, and the Department of Pathology, Faculty of Medicine, Kyushu University. These tissues had been preserved in 4% formaldehyde solution after resection. Cases 1 and 2 had died in 1969, the year after the outbreak of Yusho, while case 3 had died in 1972. From the severity of dermal lesions, case 2 had been identified as grade 4 according to the criteria established by Goto & Higuchi (1969) and case 3 as grade 3, while case 1 was judged to be grade 3 or 4 from an examination of his clinical records. The similarly preserved adipose tissue and livers of two persons without Yusho who died in accidents were also obtained for analysis from the Department of Forensic Medicine, Faculty of Medicine, Kyushu University.

Chemicals. *n*-Hexane, ethanol, potassium hydroxide, anhydrous sodium sulphate and silica gel, all reagent-grade, were purified by the methods described previously (Nagayama *et al.* 1975). Reagent-grade carbon tetrachloride and methylene chloride were fractionally redistilled. Alumina for column chromatography (about 300 mesh) was obtained from Wako Pure Chemical Industry, Ltd., Osaka, and activated by heating at 150°C for 12 hr before every use. PCDF for use as a standard was synthesized by chlorination of dibenzofuran (Nishizumi, Kuratsune & Masuda, 1975) and consisted mainly of tetra- and pentachlorodibenzofurans. 2,3,7,8-Tetrachlorodibenzofuran was kindly donated by Dr. J. G. Vos, National Institute for Public Health, Bilthoven, The Netherlands, and by Dr. I. H. Pomerantz, Food and Drug Administration, Washington, DC, USA. KC-500 was supplied by Kanegafuchi Chemical Industry, Japan.

Analytical procedure. The official standard analytical method for PCB established by the Ministry of Health and Welfare, Tokyo (Kawashiro *et al.* 1972) and the separation method for PCDFs and PCBs (Nagayama *et al.* 1975) were used to fractionate PCBs and PCDFs from tissue samples and to separate the PCBs and PCDFs in the fraction. Samples (about 5–180 g) were first homogenized with *n*-hexane (150–400 ml) and anhydrous sodium sulphate (20 g) in a Waring blender. The *n*-hexane solutions were pooled and evaporated to dryness to yield fatty residues, which were saponified with 1 N-KOH–ethanol

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solution (50 ml). The *n*-hexane extract of the KOH–ethanol solutions was concentrated and then chromatographed on a column of silica gel (2 g) eluted with *n*-hexane (150 ml). The *n*-hexane eluate containing PCBs and PCDFs was further fractionated on a column of activated alumina (3 g) using *n*-hexane–carbon tetrachloride (4:1, v/v; 60 ml) and *n*-hexane–methylene chloride (4:1, v/v; 20 ml) as successive eluants. PCBs were expected to be contained in the former eluate and PCDFs in the latter. These eluates were evaporated, and the residues were redissolved in *n*-hexane and subjected first to gas chromatography (ECD–GC) in a Beckman GC 72-5 fitted with an electron-capture detector and a glass column (2 mm × 2 m) containing Chromosorb W AW-DMCS (100–120 mesh) coated with 2% SE-30 (column temp. 200°C, carrier gas helium, flow rate 30 ml/min) and subsequently to gas chromatography–mass spectrometry (GC–MS) using a type D-100 instrument (Japan Electron Optics Laboratory, Tokyo) and a column similar to that used for ECD–GC, the ionizing energy being 25 eV and the ionizing current 0·3 mA.

PCBs were estimated quantitatively by means of the official standard analytical method already mentioned (Kawashiro *et al.* 1972), while PCDFs were estimated by comparing the heights of the gas-chromatographic peaks with those of synthesized PCDF, the assumption being made that all the PCDF isomers isolated from tissue samples had the same peak height sensitivity regardless of the number and sites of chlorine substitution. This assumption can be justified on two counts. First we had found in quantitative studies of PCDF in Kanechlors and 'Yusho oil' that the use of this assumption gave figures very close to those obtained by the perchlorination method (Nagayama *et al.* 1975 & 1976). The latter method could not be used in the present analyses because the PCDF levels in the tissue samples were much lower than those in Kanechlors or 'Yusho oil'. Secondly, as will become apparent later, the patterns of gas-chromatographic peaks were similar for PCDFs isolated from a variety of tissues taken from the Yusho patients, indicating that a fairly accurate comparison could be made of PCDF levels in various tissue on the basis of this assumption.

RESULTS

Gas chromatograms of PCB and/or PCDF fractions of the liver of a patient (case 1) and of the toxic rice oil, before and after separation on an alumina column, are shown in Fig. 1, chromatograms A, B and C indicating that a good separation of PCB and PCDF was achieved.

Comparison of chromatograms C and D shows that there is a distinct difference in the peak pattern between PCDF obtained from the liver of the patient and that from the toxic rice oil. The former consisted almost exclusively of peaks 4′, 6′ and 7′, which coincided exactly in retention times with peaks 4, 6 and 7, respectively, in chromatogram D. Peak 3 of chromatogram D had exactly the same retention time as that of 2,3,7,8-tetrachlorodibenzofuran, but was hardly seen in chromatogram C. A similar lack of peaks corresponding to peaks 1, 2 and 5 was also noted in chromatogram C.

Each peak in chromatogram C was examined by GC–MS and the components of peaks 4′ and 6′ were identified as pentachlorodibenzofurans, and that of peak 7′ as hexachlorodibenzofuran. The mass spectrum of peak 6′ is shown in Fig. 2, together with the mass spectrum of a synthesized pentachlorodibenzofuran, which showed the same retention time as peak 6′. These two mass spectra are considered to be practically the same. The gas chromatograms of other tissue samples of Yusho patients were almost the same as chromatogram C in Fig. 1.

Table 1 summarizes the results of analyses of various tissues from patients and from accident victims. With the exception of the spleen of case 2, all the tissues taken for analysis from Yusho patients contained PCDFs. The adipose tissues and livers of the accident victims contained PCBs but did not contain any detectable amount of PCDFs (detection limit 0·05 ppb). Table 1 also shows another interesting fact

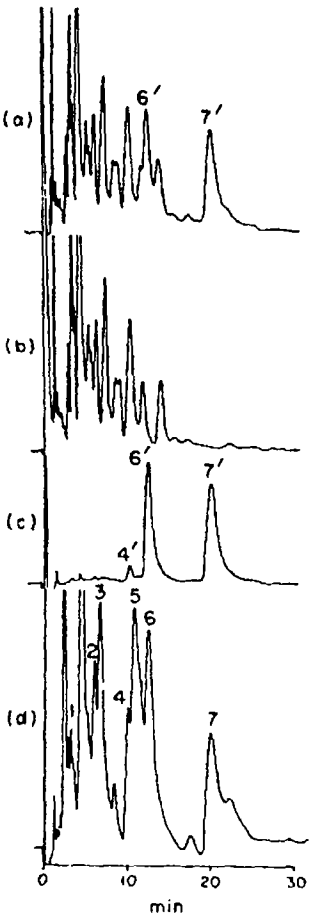

Fig. 1. Gas chromatograms of PCB and/or PCDF fractions obtained from the liver of a patient with Yusho (case 1) and from the toxic rice oil: (a) PCB + PCDF fraction from the liver of a patient with Yusho; (b) PCB fraction separated from A; (c) PCDF fraction separated from A; (d) PCDF fraction from the rice oil. The peaks were identified by GC–MS as (1) $C_{12}H_5OCl_3$; (2–3) $C_{12}H_4OCl_4$; (4–6, 4′, 6′) $C_{12}H_3OCl_5$; (7, 7′) $C_{12}H_2OCl_6$. Column, 2% SE-30; detector, ECD.

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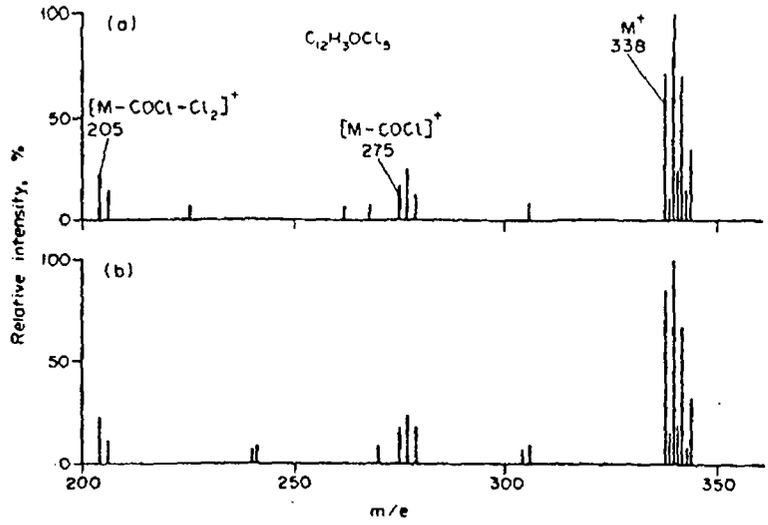

Fig. 2. Mass spectra of (a) gas-chromatographic peak 6′ of PCDF fraction from the liver of a patient (case 1) with Yusho and (b) peak 6 of synthesized PCDF.

in that in all three patients concentrations of PCDF in relation to PCB were much higher in the liver than in the adipose tissue. The concentrations of PCDF on whole basis were particularly high in the liver in cases 1 and 2. This pattern of distribution contrasted sharply with that of the PCBs which showed far higher concentrations in the adipose tissue than in the liver. Analysis of the kidneys from cases 2 and 3 showed that PCDF concentrations in this organ were very much lower than those found in the liver.

DISCUSSION

In contrast to the situation with PCBs (Burse, Kimbrough, Villanueva, Jennings, Linder & Sovocool, 1974; Curley, Burse, Grim, Jennings & Linder, 1971; Grant, Phillips & Villeneuve, 1971; Platonow, Liptrap & Geissinger, 1972), the tissue distribution of PCDFs has received little attention. This study seems to be the first to identify PCDFs in the tissues of patients with Yusho and to disclose the peculiarity of their tissue distribution, although no definite conclusions can yet be drawn from our findings because of the limited number of observations made. It is noteworthy, in this connexion, that polychlorinated dibenzo-*p*-dioxins, which are closely related to PCDFs in chemical structure, also have a definite tendency to accumulate in the livers of animals (Allen, Van Miller & Norback, 1975; Firestone, Flick, Ress & Higginbotham, 1971; Norback, Engblom & Allen, 1975; Van Miller, Marlar & Allen, 1976; Williams, Cunningham & Blanchfield, 1972). As indicated ear-

Table 1. *Concentrations of PCB and PCDF in the tissues of male patients with Yusho and of male accident victims not affected by the disease*

Case no.	Age (yr)	Time of death	Tissue	Conc (ppm) of PCB: In whole tissue	PCB: Calc on tissue fat	PCDF: In whole tissue	PCDF: Calc. on tissue fat	$\frac{\text{PCDF concn}}{\text{PCB concn}} \times 100$
				Yusho patients				
1	13	July 1969	Adipose	1·4	3·4	0·013	0·03	0·9
			Liver	0·05	4·7	0·025	2·3	50
2	25	July 1969	Adipose	1·3	8·5	0·006	0·04	0·5
			Liver	0·06	5·6	0·010	1·1	17
			Kidney	0·13	6·6	0·0005	0·03	0·4
			Spleen	0·02	8·5	ND	ND	—
3	46	May 1972	Adipose	1·2	2·1	0·007	0·001	0·6
			Liver	0·03	3·5	0·003	0·3	10
			Kidney	0·03	1·4	0·0002	0·007	0·7
			Spleen	0·02	1·9	TR	TR	—
			Lung	0·01	2·2	TR	TR	—
				Accident victims				
4	52	March 1975	Adipose	1·0	1.4	ND	ND	—
			Liver	0·08	1·3	ND	ND	—
5	58	March 1975	Adipose	0·40	0·7	ND	ND	—
			Liver	0·02	1·0	ND	ND	—

TR = 0·00005–0·0001 ppm ND = None detectable (< 0·00005 ppm)

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lier, all the three cases analysed were in the early convalescent stage as far as the Yusho incident was concerned, and the current distribution of residual PCDFs and PCBs in the bodies of survivors more than 8 years after the poisoning may differ considerably from that observed in this study. It is highly desirable that the current distribution pattern should be clarified soon, because it is closely connected with the medical treatment of the surviving patients.

In contrast to cases 1 and 2, fairly accurate information is available concerning the consumption of toxic rice oil by case 3. From an earlier epidemiological study (T. Yoshimura, unpublished data, 1973), case 3 is known to have consumed approximately 560 ml of a canned rice oil containing about 1000 ppm PCB (KC-400) and 5 ppm PCDF over a period of 140 days. Therefore, the total amounts of PCB and PCDF ingested are estimated to have been approximately 560 and 2·8 mg, respectively. On the other hand, it is calculated that the whole liver of patient no. 3, weighing 2070 g at autopsy, contained 62 μg PCB and 6·2 μg PCDF. Thus, 0·22% of the PCDF ingested apparently remained in the liver of this patient when he died in May 1972, approximately 44 months after use of the toxic rice oil had been discontinued. The corresponding figure for PCB was 0·011%, much smaller than that for PCDF. At the present time, it is hard to comment with any certainty on the possible significance of these figures, because adequate information on the intestinal absorption rate and toxicity of PCDF in man and the possible metabolic conversion of PCBs to PCDFs is lacking. It may be said at least, however, that PCDFs deserve more attention in attempts to improve our understanding of Yusho.

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