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https://doi.org/10.15017/18754

出版情報：福岡醫學雜誌. 94 (5), pp.148-157, 2003-05-25. 福岡医学会
バージョン：
権利関係：

Fukuoka Acta Med. 94(5): 148–157, 2003

New Protocol of Dioxins Analysis in Human Blood

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Abstract We developed a new analytical method for accurately determining concentrations of polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and non-ortho-coplanar polychlorinated biphenyls (Co-PCBs) at a blood volume of 5 g. The method consists of three major steps, the extraction of lipid from human blood by an accelerate solvent extractor (ASE) system, a clean-up procedure at a scale one-fourth that of the conventional method, and a sensitive determination method by a high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS) equipped with a solvent cut large volume (SCLV) injection system as a large volume injection technique. By the improvement of the pretreatment method, the operation time and consumption of the solvent could be drastically reduced, and the sensitivity of the GC-MS with the SCLV injection system was increased to 10 times the level of the classical method. From the results of the PCDDs, PCDFs and Co-PCBs concentrations measured by the developed method and the conventional method using the same blood sample, it was confirmed that the measurement became possible at a blood volume of 5 g. The developed method is more effective than the conventional method for treating many samples within a short period of time with high reproducibility.

Introduction

The Yusho poisoning accident, which affected over 1800 persons, occurred in 1968 in western Japan, and was caused by the ingestion of cooking rice oil which contained contaminated PCBs, PCDFs and PCDDs. Over 30 years have passed since the outbreak of Yusho, and almost all of the Yusho patients' peculiar symptoms have improved. However, some patients are still afflicted with subjective symptoms. These patients even now have a much higher concentration of PCDFs in their blood than do unaffected persons[2)3)7)]. Therefore, a follow-up study of PCDDs, PCDFs, and Co-PCBs concentrations in the blood of Yusho patients is very important when considering the health care of these patients. We have been determining the concentrations of PCDDs, PCDFs and Co-PCBs in the blood samples collected from Yusho patients in order to establish new Yusho criteria[2)3)7)]. In the conventional measuring method, 20-50 ml of blood is needed to exactly measure the PCDDs, PCDFs and Co-PCBs concentrations. However, since most patients are now over 60 years old, collecting blood in this amount is restricted. These patients can safely supply only small volumes of blood for the measurement of PCDDs, PCDFs and Co-PCBs concentrations. Therefore, to reduce the physical burden on patients, it is neces-

sary to develop a highly sensitive analytic method that can accurately evaluate PCDDs, PCDFs and Co-PCBs concentrations from 5 g blood samples. In addition, since the extraction procedure of PCDDs, PCDFs and Co-PCBs from the blood by the conventional method is very complicated and time-consuming, it is not a suitable procedure for processing many samples.

In this study, we developed an analytic method to measure the PCDDs, PCDFs and Co-PCBs concentrations in 5 g blood samples using a HRGC/HRMS equipped with a Solvent Cut Large Volume (SCLV) injection system as a large volume injection technique. We also developed and examined efficient methods to speed up the pretreatment procedure for blood samples and to reduce the background levels such that they do not affect the measurement of the PCDDs, PCDFs and Co-PCBs.

Materials and Methods

Materials.

Native PCDDs, native PCDFs and native Co-PCBs, as authentic standards, were purchased from Wellington Laboratories, Ontario, Canada. [$^{13}C_{12}$]-PCDDs, [$^{13}C_{12}$]-PCDFs and [$^{13}C_{12}$]-PCBs, as internal standards, were also purchased from Wellington Laboratories, Ontario, Canada. An active carbon column was prepared as follows: active carbon was purchased from Nacalai Tesque, Kyoto, Japan, refluxed five times with toluene for 5 hr, and dried in vacuum, after which 500 mg of the active carbon was mixed with 500 g of anhydrous sodium sulfate (Wako Pure Chemicals Ind, Co. Ltd., Tokyo, Japan). A silver nitrate/silica gel was purchased from Wako Pure Chemicals Ind. Co. Ltd., Tokyo, Japan. Distilled water used in this experiment was treated with n-hexane. All other chemicals used were of the analytical grade of PCB and phthalate commercially available.

Sample preparation.

Lipid was extracted from the blood samples by an accelerated solvent extractor (ASE-200, Dionex, Sunnyvale, CA). Each blood sample was accurately weighted to 5 g and mixed with 4 g Isolute (International Sorbent Technology Ltd., Hengoed, Mid Glamorgen, UK). After the mixed sample was loaded into the extraction cell, [$^{13}C_{12}$]-PCDDs, [$^{13}C_{12}$]-PCDFs and [$^{13}C_{12}$]-PCBs, as internal standards, were added. The following programmed parameters were used for these extractions: a pressure of 2000 psi and a temperature of 150°C, with a static time of 10 minutes, a flushing volume of 50 ml, 90 seconds purging, a 60% flushing volume for two cycles, and acetone : n-hexane (1 : 4, v/v) as the extraction solvent. The extract was evaporated to near dryness after being treated with anhydrous sodium sulfate (10 g), and the lipid contents were determined gravimetrically. The extracted lipid carried out the cleanup at a scale one-fourth that of the conventional method[2)3)7)]. More specifically, the lipid was dissolved in n-hexane and treated with concentrated sulfuric acid. The separated hexane layer was applied to a silver nitrate/silica gel column (0.5 g) and eluted with 15 ml of hexane. The eluted solution was loaded to an active carbon column (0.5 g) after being evaporated to 1 ml and separated into two fractions. The first fraction containing mono-ortho-choline substituted biphenyls (mono-ortho-PCBs) was eluted with 10 ml of dichloromethane/n-hexane (1 : 9, v/v). PCDDs, PCDFs and non-ortho-PCBs were eluted with 25 ml of toluene as the second fraction. The eluate was evaporated to near dryness and transferred to an

injection vial, and the syringe standard was added. The column packing (silver nitrate silica gel, active carbon column and anhydrous sodium sulfate) used in this experiment was washed by ASE-200 under the same conditions as the lipid extraction with n-hexane or toluene.

Analysis of PCDDs, PCDFs and Co-PCBs.

Concentrations of the PCDDs, PCDFs and Co-PCBs were measured using HRGC/HRMS equipped with an SCLV injection system. The analytical conditions were as follows: the gas chromatograph was an HP-6890 A series (Hewllet-Packard, Palo Alto, CA)equipped with an Autospec Ultima E, (Micromass Ltd., Manchester, UK) and a solvent cut large-volume injection system (SCLV), SGE international., Victoria, Australia; the column used was an BPX-5 fused silica pre-capillary column, 0.25 mm i.d. 6 m, 0.25μm film thickness (SGE International., Victoria, Australia); the analytical column, 0.15 mm i.d. × 30 m, 0.15μm film thickness (SGE International., Victoria, Australia); the column was heated from 80 °C to 320 °C at a rate of 20 °C / min, maintained at 320 °C for 5 min, cooled to 180 °C at a rate of 70 °C / min, maintained at 180 °C for 1 min, heated to 320 °C at a rate of 5 °C / min, and then maintained at 320 °C for 5 min. The injection temperature and ion source temperature were maintained at 280 °C and 270 °C, respectively, and the carrier gas (helium) flow rate (constant flow) was 1.3 ml/min. The ionizing current, ionizing energy, accelerating voltage, and trap current were 550 mA, 40 eV, 7.8 kV and 750 mA, respectively. PCDDs, PCDFs and Co-PCBs were analyzed in a single ion record mode. The resolution was maintained at 10000 at 10%. Analysis of tetrachlorodibenzo-p-dioxins (TCDDs), pentachlorodibenzo-p-dioxins (PeCDDs), hexachlorodibenzo-p-dioxins (HxCDDs), heptachlorodibenzo-p-dioxins (HpCDDs) and octachlorodibenzo-p-dioxin (OCDD) involved the use of [$^{13}C_{12}$]-2, 3, 7, 8-TCDD, [$^{13}C_{12}$]-1, 2, 3, 7, 8-PeCDD, [$^{13}C_{12}$]-1, 2, 3, 4, 7, 8-HxCDD, [$^{13}C_{12}$]-1, 2, 3, 6, 7, 8-HxCDD, [$^{13}C_{12}$]-1, 2, 3, 7, 8, 9-HxCDD, [$^{13}C_{12}$]-1, 2, 3, 4, 6, 7, 8-HpCDD, and [$^{13}C_{12}$]-1, 2, 3, 4, 6, 7, 8, 9-OCDD as internal standards, respectively. The analysis of tetrachloro-dibenzofurans (TCDFs), pentachlorodibenzofurans (PeCDFs), hexachlorodibenzofurans (HxCDFs), heptachlorodibenzofurans (HpCDFs) and octachlorodibenzofuran (OCDF) involved the use of [$^{13}C_{12}$]-2, 3, 7, 8-TCDF, [$^{13}C_{12}$]-1, 2, 3, 7, 8-PeCDF, [$^{13}C_{12}$]-2, 3, 4, 7, 8-PeCDF, [$^{13}C_{12}$]-1, 2, 3, 4, 7, 8-HxCDF, [$^{13}C_{12}$]-1, 2, 3, 6, 7, 8-HxCDF, [$^{13}C_{12}$]-1, 2, 3, 7, 8, 9-HxCDF, [$^{13}C_{12}$]-2, 3, 4, 6, 7, 8-HxCDF, [$^{13}C_{12}$]-1, 2, 3, 4, 6, 7, 8-HpCDF, [$^{13}C_{12}$]-1, 2, 3, 4, 7, 8, 9-HpCDF, and [$^{13}C_{12}$]-1, 2, 3, 4, 6, 7, 8, 9-OCDF as internal standards, respectively. The analysis of 3, 3', 4, 4'-tetrachlorobiphenyl (TCB), 3, 4, 4', 5-TCB, 3, 3', 4, 4', 5-pentachlorobiphenyl (PeCB) and 3, 3', 4, 4', 5, 5'-hexachlorobiphenyl (HxCB) involved the use of [$^{13}C_{12}$]-3, 3', 4, 4'-TCB, [$^{13}C_{12}$]-3, 4, 4', 5-TCB, [$^{13}C_{12}$]-3, 3', 4, 4', 5-PeCB and [$^{13}C_{12}$]-3, 3', 4, 4', 5, 5'-HxCB as internal standards, respectively. [$^{13}C_{12}$]-1, 2, 3, 4-TCDD was used as a syringe spike.

Results and Discussion

Because PCDDs, PCDFs, and Co-PCBs are found at low concentrations in a 5 g blood sample, a highly sensitive technique is needed. The response of the analytical system can be increased with the introduction of a large volume of the final extract into the GC/MS. Though several large-

volume injection techniques have been proposed for increasing the sensitivity of GC/MS, a technique using a Solvent Cut Large-Volume (SCLV) injection system has been reported to be the most useful for analysis of PCDDs, PCDFs and Co-PCBs in human blood[4]. This method consists of a two-stage chromatography system, the injected large-volume samples in the GC/MS being separated the solvent and the analytic compound by the pre-column to remove the various interfering substances in the sample. The solvent containing the various interfering substances is then vented to waste through a solvent cut valve. The analytic compounds are focused at the head of the analytical column by a cold trap, and the analytical column is then heated by temperature program, and the analytic compounds are separated and determined. Because most matrices in an injected sample can be removed by pre-column, the analytical column used can be a narrow bore (0.1-0.15 mm) with a thin film thickness (1.0-1.5 μm). Consequently, extremely narrow peaks are obtained, and the mass sensitivity is increased. By using HRGC/HRMS with a SCLV injection system, the sensitivity can be increased to 10 times the level of the classical method (Fig. 1-2). It therefore seemed possible that concentrations of PCDDs, PCDFs and Co-PCBs could be measured at a blood volume of 5 g.

Sample-extraction procedures are often perceived as bottlenecks in analytical methods. Because the procedure for extracting lipids from the blood by conventional methods is very complicated and time-consuming, it is not suitable procedure for processing many samples. An accelerated solvent extractor (ASE), which employs a new extraction procedure using organic solvents at high pressures and temperatures above the boiling point, is widely used to replace Soxhlet or liquid-liquid extraction for extraction of PCDDs, PCDFs and Co-PCBs from environmental samples[1)5)6)8)]. The ASE technique is expected to make possible lipid extraction from blood in short periods of time with a small solvent volume as compared to conventional methods. We investigated the extraction of lipids from a blood sample by an ASE at a pressure of 2000 psi and a temperature of 150 °C using a 5 g blood sample. The results are summarized in Table 1. When lipids were extracted from the blood by an ASE-200 with the acetone/n-hexane system solvent containing over 20% acetone, the lipid content was nearly that resulting from the conventional method. With regard to the concentrations of PCDDs, PCDFs and Co-PCBs, the developed method using acetone : n-hexane (1 : 4, v/v) as a extraction solvent resulted in almost the same values as with the conventional method. These finding indicate that the ASE technique shows good potential regarding the recovery of lipids from blood.

Furthermore, the lipid content extracted by the developed method and the conventional method using 20 g samples of the same bloods were compared for blood samples collected from eight normal subjects. The results are shown in Fig. 3. It was confirmed that the developed method could produce the same lipid content as the conventional method. The concentrations of each isomer of PCDDs, PCDFs and Co-PCBs by both methods were also nearly the same, and the total toxicity equivalency quantity (TEQ) levels were almost equal to those obtained by the conventional method. In addition, the developed method demonstrated high reproducibility based on experiments conducted using the same blood sam-

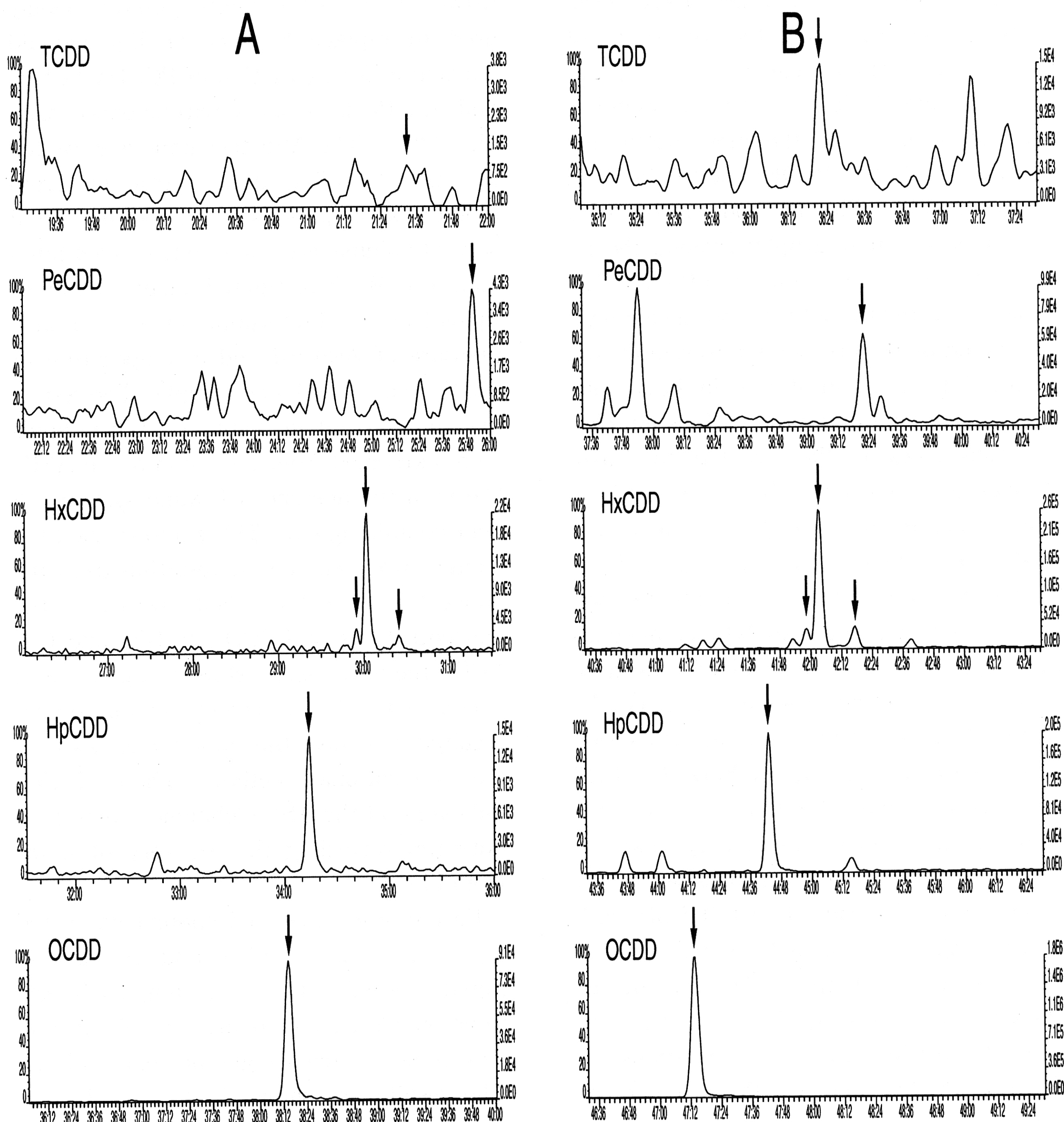

Fig. 1 High-resolution gas chromatography/high-resolution mass spectrometry chromatograms of PCDDs in the blood.
A, classical injection (2 μl/5 μl); B, SCLV injection (10 μl/25 μl).

ple (Fig. 4). Moreover, recovery of the ^{13}C-labeled internal standard was 60% overall when the concentrations of PCDDs, PCDFs and Co-PCBs in the blood of 19 Yusho patients were measured by the developed method (Table 2). These finding indicate that the developed method is essentially equivalent to the conventional method.

Furthermore, the developed method has the following important advantages: (1) the extraction of lipids from blood can be done automatically by the ASE technique, and one sample can be treated within 30 min; (2) the volume of the used solvent can be

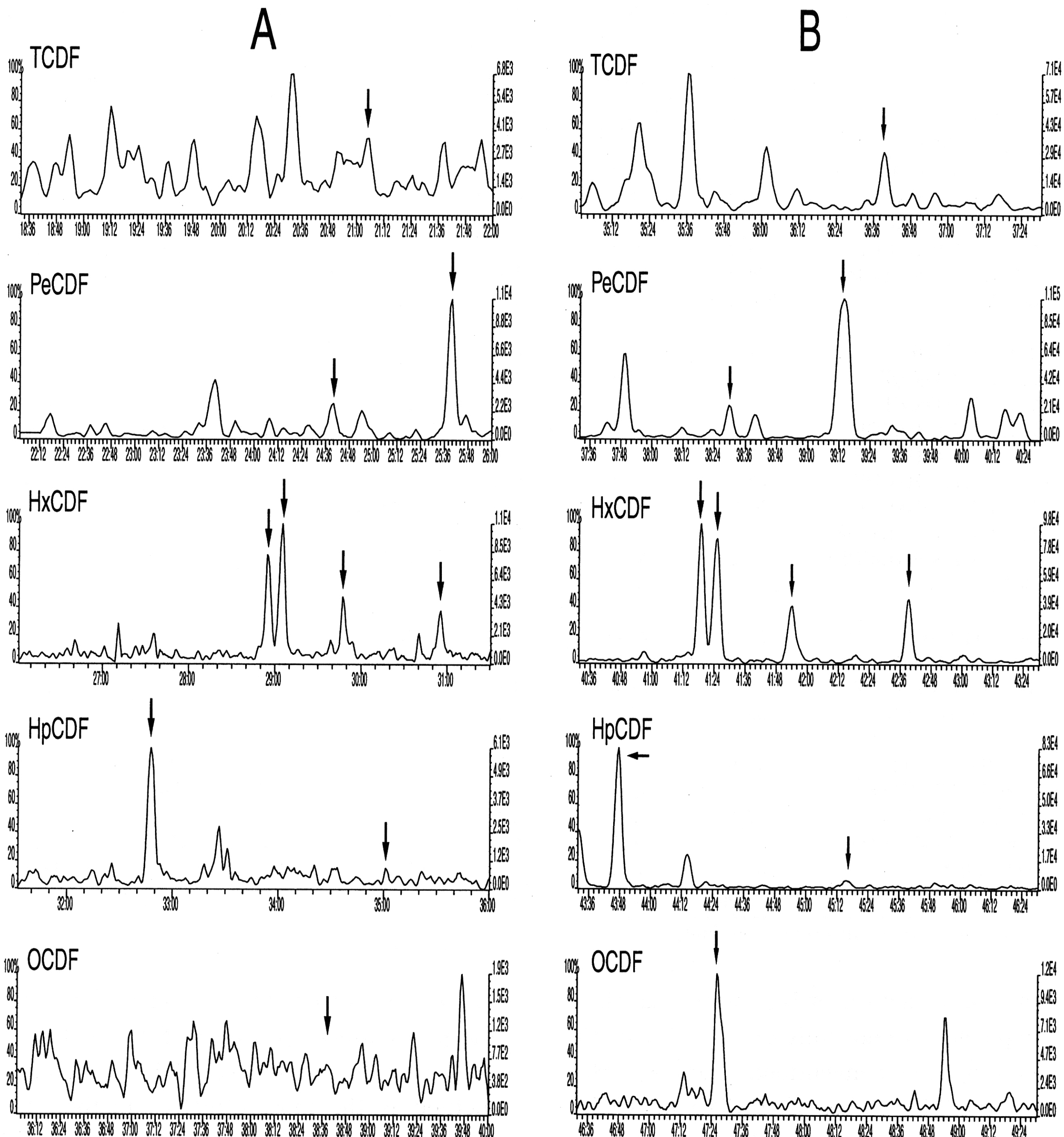

Fig. 2 High-resolution gas chromatography/high-resolution mass spectrometry chromatograms of PCDFs in the blood.
A, classical injection (2 μl/5 μl); B, SCLV injection (10 μl/25 μl).

reduced to one-quarter or more that of the conventional method by the simplified clean-up procedure; and (3) the improvement of the pretreatment method makes it possible to sufficiently reduce background levels such that they do not affect the measurement of PCDDs, PCDFs and Co-PCBs. However, when HpCDD and OCDD in blood samples are extracted by an ASE, they show high concentrations compared with those obtained by the conventional method. Although the cause of this phe-

Table 1 Extraction of PCDDs, PCDFs and Co-PCBs from blood samples by ASE-200

Congeners	Concentration (pg/gLipid)						
	Conventional method	Acetone : Hexane (2 : 1)		Acetone : Hexane (1 : 4)		Hexane	
		Mean (n=3)	SD	Mean (n=3)	SD	Mean (n=3)	SD
2,3,7,8-TCDD	2.4	1.8	0.7	2.1	0.4	4.2	1.5
1,2,3,7,8-PeCDD	8.7	10.8	1.5	8.9	1.4	20.1	6.7
1,2,3,4,7,8-HxCDD	4.9	4.2	0.2	3.7	1.3	8.3	2.3
1,2,3,6,7,8-HxCDD	34.1	32.3	2.7	31.6	0.8	71.6	9.4
1,2,3,7,8,9-HxCDD	5.2	6.7	0.7	5.2	0.8	14.5	3.2
1,2,3,4,6,7,8-HpCD	28.9	43.1	1.4	35.1	2.2	78.3	15.6
OCDD	247.5	567.4	34.1	395.3	30.6	764.7	149.3
2,3,7,8-TCDF	1.4	2.8	0.4	2.1	0.2	4.9	0.9
1,2,3,7,8-PeCDF	1.4	1.4	0.3	1.3	0.4	2.8	1.0
2,3,4,7,8-PeCDF	18.1	19.1	2.4	18.6	3.3	41.3	6.3
1,2,3,4,7,8-HxCDF	8.4	9.7	0.8	13.3	7.9	19.8	1.7
1,2,3,6,7,8-HxCDF	7.3	7.8	0.7	7.8	2.0	15.9	3.6
2,3,4,6,7,8-HxCDF	2.8	3.1	0.2	3.0	1.0	6.3	1.4
1,2,3,7,8,9-HxCDF	N.D.	2.5	0.4	2.6	0.5	5.0	1.7
1,2,3,4,6,7,8-HpCDF	4.2	5.8	0.8	4.8	0.6	10.6	1.1
1,2,3,4,7,8,9-HpCDF	N.D.	N.D.		N.D.		N.D.	
OCDF	N.D.	N.D.		N.D.		5.2	3.3
344'5-TCB (#81)	N.D.	N.D.		N.D.		11.2	2.5
33'44'-TCB (#77)	10.8	35.9	7.4	28.0	0.3	38.2	8.2
33'44'5-PenCB (#126)	82.8	81.8	4.6	75.0	2.6	168.4	27.3
33'44'55'-HxCB(#169)	58.8	54.8	2.3	54.1	3.1	136.3	23.4
TotalPCDDs-TEQ	15.9	17.4	0.7	15.4	1.5	34.5	7.1
TotalPCDFs-TEQ	11.1	12.3	1.3	12.3	2.7	26.1	4.1
TotalPCDDs/PCDF	27.0	29.7	2.0	27.7	4.1	60.6	11.2
TotalCo-PCBs-TEQ	8.9	8.7	0.5	8.0	0.3	18.2	3.0
TotalTEQ	35.9	38.4	2.5	35.8	4.4	78.8	14.1
Lipid(%)	0.290	0.290	0.017	0.297	0.006	0.117	0.015

N.D., less than the determination limit.

nomenon is unclear, it is possible that these compounds are not fully extracted from the blood by the conventional method. But, since the TEF values of HpCDD and OCDD are small, at 0.01 and 0.0001, respectively, they do not significantly influence the total TEQ values on the lipid base. Nevertheless, this details need to be clarified. Another remaining problem that must be addressed is the high background values of 3, 3', 4, 4'-TCB (PCB 77), making it difficult to precisely measure the concentration.

In conclusion, an analytic method for measuring concentrations of PCDDs, PCDFs and Co-PCBs in blood samples as small as 5 g, and an efficient method for speeding up the pretreatment procedure for blood samples can be developed. The developed method is the most effective method for accurate measurement of PCDD, PCDF and Co-PCB concentrations using a blood volume of only 5 g. Moreover, this method allows many samples to be treated in a short period with high reproducibility in comparison with the conventional method. Using this method, we measured the concentrations of PCDDs, PCDFs and Co-PCBs in blood samples collected from 78 Yusho patients living in Fukuoka Prefecture in 2001.

Table 2 Recoveries of PCDDs, PCDFs and Co-PCBs by the dev

Congeners	Recovery (n=19) Mean	SD
^{13}C-2,3,7,8-TCDD	69.0	3.3
^{13}C-1,2,3,7,8-PeCDD	81.4	7.1
^{13}C-1,2,3,4,7,8-HxCDD	92.5	11.2
^{13}C-1,2,3,6,7,8-HxCDD	85.0	7.4
^{13}C-1,2,3,7,8,9-HxCDD	85.8	5.6
^{13}C-1,2,3,4,6,7,8-HpC	87.6	7.1
^{13}C-OCDD	81.7	12.0
^{13}C-2,3,7,8-TCDF	91.6	3.9
^{13}C-1,2,3,7,8-PeCDF	78.2	4.8
^{13}C-2,3,4,7,8-PeCDF	76.1	5.2
^{13}C-1,2,3,4,7,8-HxCDF	81.2	5.9
^{13}C-1,2,3,6,7,8-HxCDF	79.0	4.5
^{13}C-2,3,4,6,7,8-HxCDF	95.9	4.8
^{13}C-1,2,3,7,8,9-HxCDF	95.8	7.8
^{13}C-1,2,3,4,6,7,8-HpC	86.6	6.3
^{13}C-1,2,3,4,7,8,9-HpC	89.2	13.9
^{13}C-OCDF	87.7	18.5
^{13}C-33'45'-TCB(# 81)	66.3	2.9
^{13}C-33'44'-TCB(# 77)	63.9	2.3
^{13}C-33'44'5-PenCB(# 126)	68.2	2.9
^{13}C-33'44'55'-HxCB(# 169)	73.5	5.6

Acknowledgment

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Health Labour and Welfare, Japan.

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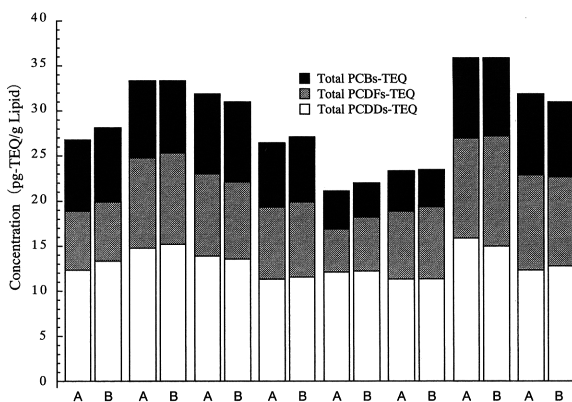

Fig. 3 Comparison of PCDDs, PCDFs and Co- PCBs concentrations in the blood by the conventional method (A) and the developed method (B).

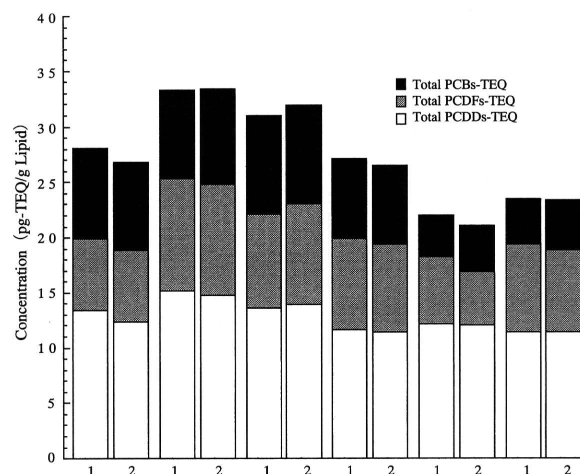

Fig. 4 Reproducibility test of the developed method.

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(Received for publication April 1, 2003)

（和文抄録）

ヒト血液中ダイオキシン類分析の超高感度ならびに迅速化に関する検討

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5 gの血液量でダイオキシン類分析を可能にする測定系および血液試料の前処理法を検討した．すなわち，高速溶媒抽出装置による血液試料からの脂質の抽出法，従来法の1/4スケールでのカラムクリーンアップ法および大量溶媒注入装置（SCLV injection system）を装備したHRGC/HRMSを用いて高感度測定の一連の分析方法について検討し，超高感度迅速分析法を確立した．大量溶媒注入装置の装備によりHRGC/HRMSの相対感度は10倍程度向上した．同一の血液を用いて行った従来法との比較検討結果から，5 gの血液量でもダイオキシン類濃度が十分測定可能であることを確認された．さらに，従来法に比べ血液試料の前処理段階で費やす時間を大幅に短縮することができた．本法は5 g程度の少量の血液量でダイオキシン類を測定できるだけでなく，多数のサンプルを効率よく，かつ迅速に処理するのに効果的な方法である．