EFFICIENCY OF FOCUSED MICROWAVE-ASSISTED EXTRACTION OF PCDDS / PCDFS FROM HUMAN LIVER SAMPLES

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Introduction

Information about human exposure to dioxins based on blood levels is accumulating, however, data from human samples, such as autopsy samples are still limited. The analytical procedures for PCDDs/PCDFs are composed of three steps; extraction, clean-up and detection. Tremendous efforts have been made to improve of the clean-up process and detection procedure by developing high resolution GC/MS. For extraction, a classic method, the liquid-solid extraction method, is usually used. The extraction process is simple, though, tedious and time consuming, and further it has been reported that extraction only by organic solvents is not sufficient for PCDDs /PCDFs.[1, 2)] Thus, it is important to improve this process for high through-put analysis.

Recently, a new method, focused microwave-assisted extraction (FMA) has been developed. In this method, microwaves are focused on a small area with a high irradiation-power efficiency. During extraction, samples are placed in a thimble-form filter and the centrifuge process can be neglected. Therefore, it is expected that use of FMA can lead to savings in manpower and time. It has been reported that polycyclic aromatic hydrocarbons (PAH) and polychlorinated biphenyls (PCB) have been recovered from river sediment and soil with high efficiency.[3 - 5)] However, it is not known yet whether FMA is an efficient method or not for dioxin extraction.

In this study, we applied FMA to human liver samples for extraction of PCDDs/PCDFs and compared this with an acetone/hexane extraction method, an ethanol /hexane extraction method and a hexane extraction after treatment with alkali overnight.

Material and methods

Chemicals

Authentic standards of native PCDDs and PCDFs, and $^{13}C_{12}$-PCDDs and $^{13}C_{12}$-PCDFs were purchased from Wellington Laboratories (Ontario, Canada). Active carbon-impregnated silica gel of dioxin-analysis grade was purchased from Wako Pure Chemicals (Osaka, Japan). All solvents used were of dioxin-analysis grade. Ultra-pure water was supplied from a Milli-Q SP TOC system from Japan Millipore (Tokyo, Japan).

Human sample

Twelve autopsy cases were provided. All cases were autopsied within 2 hours after death. About 5 g of liver from each case was stored in deep freeze until analysis. Permission for

About 5 g of liver from each case was stored in deep freeze until analysis. Permission for analyzing dioxins was obtained from the bereaved families.

Preparation of samples for analysis

The above samples, 1.5 g of liver from each case, of the 12 cases were pooled. Samples were spiked with $^{13}C_{12}$-PCDDs and $^{13}C_{12}$-PCDFs as internal standards. The pooled sample, 18 g, was homogenized in a mortar in the presence of the same amount of Na_2SO_4 [6]. A three gram aliquot was used for each extraction, A,B,C and D. Method A: the extraction thimble in which the sample was placed was put in the extraction tube of a SOXWAVE 3.6 (PROLABO, Briare, France), 50 ml of acetone/hexane (2:1 v/v) added, and extracted under 200 w electric power for 30 min. Method B: the sample was shaken after addition of 50 ml of acetone/hexane (2:1 v/v) [7] for 30 min and centrifuged at 2,000 rpm for 10 min. Method C: the procedure was the same as method B, except for ethanol/hexane (1:3 v/v) instead of acetone/hexane. Method D: the sample had 100 ml of 1N KOH added to make a final concentration of 0.9-1N and was then shaken for 30 min and stood overnight at room temperature. To this mixture, 100 ml of *n*-hexane was added, shaken for 30 min, and then centrifuged at 2,000 rpm for 10 min. These extraction processes were performed in triplicate. These extraction processes were repeated a further two times in all the methods, A-D. The extracts were pooled and washed with ultra-pure water. The *n*-hexane layers were dried over anhydrous sodium sulfate, evaporated to dryness, and the lipid residues were weighed, except for method D. The residues were dissolved in two ml of *n*-hexane and applied to a multi-layer column composed of 10% silver nitrate-silica gel, 22% sulfuric acid-silica gel, 44% sulfuric acid-silica gel, and 2% potassium hydroxide-silica gel [8]. 150 ml of *n*-hexane was passed through the column and the effluent was evaporated. The remaining concentrate was applied to an active carbon-impregnated silica gel column[9], washed with 200ml of 25% (v/v) dichloromethane/ *n*-hexane, then eluted with 200 ml of toluene. The eluent, toluene was evaporated to 2 to 3 ml by a rotary evaporator and then spontaneously at room temperature to almost empty in a vessel. Five *ul* of *n*-nonane containing $^{13}C_{12}$-1,2,3,4-TCDD and $^{13}C_{12}$-1,2,3,7,8,9- HxCDD spiking substances were added to this vessel.

Analysis

A GC/MS system, which consisted of an HP-6890A gas chromatograph (Hewllet-Packard, Palo Alto, California, U.S.A.) and a Finnigan MAT-95S mass spectrometer (Finnigan MAT GmbH, Bremen, Germany) was used. The column used was a DB-5MS fused silica capillary column, 0.25 mm i.d. x 60 m, with 0.25 *um* film thickness (J&W Scientific, Folsom, California, U.S.A.). The column temperature was maintained at 140 ℃ for 1 min, heated to 220 ℃ at a rate of 17 ℃ /min, heated to 310 ℃ at a rate of 3 ℃/min, and maintained at 310 ℃ for 4 min. The injection temperature was 260 ℃, ion source temperature was maintained at 250 ℃, and the carrier gas (helium) rate was 1.2 ml/min. The ionizing current, ionizing energy and accelerating voltage were 1 mA, 60 eV and 5 kV, respectively. The resolution of the mass spectrometer was maintained at about 10,000 throughout the work, and analysis was carried out according to an SIM using 50 selected ions.

Results and discussion

Convenience of FMA

In MAE (microwave-assisted extraction), which was developed before FMA was devised, a sample and extraction solvent are enclosed in a sealed container, placed in a microwave, and

bumping, it is necessary for the container to be sealed. This made it difficult for researchers to use MAE for the extraction of dioxins. Since FMA is equipped with a reflux column, there is no difficulty to use FMA for extraction.

Efficiency of Lipid Extraction

Levels of PCDDs/PCDFs in biological samples are frequently expressed in terms of per g lipid. Therefore, the efficiency of lipid extraction is an important factor when evaluating dioxin exposure. Therefore, this was compared among the three methods, except for method D, in which lipids are degraded. The mean ± standard errors of the extracted lipid weight (w/w%) by method A, B and C were 6.77±0.09, 6.86±0.13 and 6.80±0.12%, respectively. Coefficients of variation were 2.80, 3.64 and 3.53%, respectively. There was no significant difference in recovery of lipid among the three methods, and the coefficient of variation was the smallest in A.

Efficiency of PCDDs / PCDFs Extraction

GC/MS analysis revealed that there was no significant difference in recovery of all ^{13}C-PCDDs / PCDFs congeners among methods A-D. Further, levels of the endogenous PCDDs/PCDFs in the liver sample detected by the four methods are summarized in Table 1. There was no significant difference among four methods in all congeners, either. These results clarified that the extraction efficiency of FMA is comparable to liquid-solid extraction with or without degradation of matrix by alkaline treatment. The time taken for FMA was about a half of that for liquid-solid extraction, including the time for centrifugation. Furthermore, it was revealed that the endogenous PCDDs/PCDFs were fully extracted by both FMA and liquid-solid extraction, even if the cellular matrix was not degraded. We conclude that FMA is a time saving method without any loss of precision

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Table 1. Comparison of four extraction methods by the extractable levels of PCDDs / PCDFs from human liver *

Congener	Internal standards (%)				Endogenous dioxins											
					A			B			C			D		
	A	B	C	D	Mean (pg/g)	SE (pg/g)	CV (%)	Mean (pg/g)	SE (pg/g)	CV (%)	Mean (pg/g)	SE (pg/g)	CV (%)	Mean (pg/g)	SE (pg/g)	CV (%)
2,3,7,8-TCDD	91	94	91	96	0.5	0.06	21	0.5	0.04	15	0.3	0.03	15	0.4	0.04	17
1,2,3,7,8-PeCDD	88	92	87	91	3.0	0.39	22	2.9	0.21	13	3.1	0.29	16	3.0	0.26	16
1,2,3,4,7,8-HxCDD	81	81	82	78	0.4	0.02	9.5	0.6	0.06	17	0.5	0.03	11	0.5	0.05	17
1,2,3,6,7,8-HxCDD	71	73	72	73	4.9	0.23	8.2	5.4	0.34	11	5.2	0.26	8.6	5.2	0.41	14
1,2,3,7,8,9-HxCDD	-	-	-	-	0.6	0.03	9.8	0.7	0.04	12	0.6	0.03	7.6	0.6	0.01	4.1
1,2,3,4,6,7,8-HpCDD	62	64	62	67	9.9	0.39	6.9	11	0.37	5.7	11	0.40	6.5	10	0.60	10
OCDD	60	63	62	63	68	3.64	9.3	70	1.89	4.7	70	1.38	3.4	67	2.76	7.1
2,3,7,8-TCDF	72	74	72	75	0.2	0.02	17	0.2	0.02	13	0.2	0.02	13	0.2	0.01	8.7
1,2,3,7,8-PeCDF	82	86	84	84	0.2	0.01	11	0.1	0.01	8.7	0.2	0.01	7.9	0.1	0.01	13
2,3,4,7,8-PeCDF	84	83	82	86	2.9	0.07	4.1	3.2	0.07	3.8	3.2	0.17	9.1	3.0	0.06	3.5
1,2,3,4,7,8-HxCDF	80	77	80	80	1.1	0.11	17	1.3	0.10	13	1.2	0.09	14	1.2	0.07	11
1,2,3,6,7,8-HxCDF	79	77	78	78	1.2	0.03	4.6	1.4	0.06	7.1	1.4	0.07	8.9	1.3	0.04	5.6
2,3,4,6,7,8-HxCDF	74	74	73	74	0.7	0.00	1.0	0.8	0.03	5.6	0.8	0.07	17	0.7	0.01	1.4
1,2,3,7,8,9-HxCDF	70	70	70	71	0.1	0.01	22	0.1	0.02	27	0.2	0.01	11	0.1	0.01	20
1,2,3,4,6,7,8-HpCDF	74	76	74	77	1.3	0.02	3.0	1.2	0.05	6.8	1.3	0.06	8.2	1.3	0.10	14
1,2,3,4,7,8,9-HpCDF	68	69	66	71	0.2	0.02	16	0.1	0.02	24	0.2	0.02	27	0.2	0.01	14
OCDF	-	-	-	-	0.3	0.04	19	0.4	0.05	23	0.4	0.03	16	0.3	0.03	15
Congener group-TEQ																
PCDDs-TEQ	-	-	-	-	4.2	0.36	15	4.1	0.14	5.8	4.2	0.31	13	4.1	0.17	7.4
PCDFs-TEQ	-	-	-	-	1.8	0.02	2.2	2.0	0.05	4.3	2.0	0.11	9.5	1.9	0.03	2.9
Total-TEQ	-	-	-	-	6.0	0.35	10	6.1	0.11	3.1	6.2	0.31	8.6	6.0	0.15	4.2

SE : Standard error, CV : Coefficient of variation, A : FMA, B : Acetone/hexane, C : Ethanol/hexane, D : Alkali/hexane

* Values are mean of triplicate