DEVELOPMENT OF DIOXIN ANALYSIS IN 10mL HUMAN BLOOD.

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Introduction

Dioxin (PCDDs/PCDFs, Co-PCB) causes many problems as a pollutant on the human organism. Generally dioxin analysis requires approximately 50mL of blood, and collecting large amount of blood is a big burden for the analysis subject. It's preferable to reduce the amounts of blood sample. An extraction of lipid, clean up of a sample, and reducing a blank level is necessary to improve the measurement sensitivity of GC/MS, and to reduce the amount of sample. It is important to choose the equipment to decrease the blank level, and Kitamura announced a technique with centrifuge-tube. Choice of a solvent and a clean-up technique were important, a suitable solvent should be chosen, and an efficient extraction can be expected by using multiple layer columns. It is possible to improve the sensitivity of GC/MS by using a technique that is a system of the large volume injection being developed recently. By reducing blood sample volume and analysis subjects burden, the acceptable sample for the analysis can be extended to the baby blood, the cord blood, and so on. Our aim is to reduce the amount of blood sample down to 10mL and simplify dioxins analysis by the combination of such a technique.

Methods and Materials

In house volunteers provided blood sample. A blood sample of 10g was transferred to an 80mL centrifuge-tube. Adding $^{13}C_{12}$ internal standards, the sample was shaken for 10 minutes for liquid-liquid extraction, after the addition of 6mL of saturated ammonium sulfate solution and 24ml of 25%(v/v) ethanol-hexane. The upper hexane layer was separated, and the lower layer was extracted with 20mL of hexane twice. Combined former hexane layers were rinsed by 20mL of the distilled water, and dehydrated by adding un-hydrous Na_2SO_4.

The combined hexane fraction obtained was concentrated to about 1 mL by a rotary evaporator and then dried by standing at room temperature. The extracted lipid was then weighed. The lipid was reconstituted with 1mL of hexane, and submitted for clean up.

Multiple layers column was conditioned with 100mL of hexane, and activated carbon silica gel column was conditioned with the 10mL hexane. Multiple-layer column composed of 1g of $AgNO_3$-silica gel, 0.5g of silica gel, 1.5g of 22% H_2SO_4 silica gel, 1.5g of 44% H_2SO_4 silica gel, and 0.5g of silica gel.

By passing through the column, multiple-layer column being connected to activated carbon silica gel column, the reconstituted solution was cleaned-up.1mL of the solution was applied to the column, and eluted with 25mL of hexane. Then the multiple layer column was removed.

Activated carbon silica gel column was washed with 5mL of hexane, eluted by 50mL of 25% dichloromethane/n-hexane into a pear-shaped pointed bottom flask (1^{st}.fraction to analyse for

mono-ortho Co-PCBs), and then eluted by 150mL of toluene into another pear-shaped pointed bottom flask(2nd.fraction to analyse for PCDDs, PCDFs and non-ortho Co-PCBs). Each fractions were evaporated to approximately 1mL, and the internal standard, spike in toluene, was added. The samples were evaporated to approximately 50µL under dryness flow of a nitrogen gas, and the sample of 20µL was injected into the injector to analyse by HRGC-HRMS.
The HRGC/HRMS analysis was performed on AutoSpec-Ultima high-resolution mass spectrometer, (Micromass, UK) coupled to an Agilent 6890 Plus GC (Agilent Technologies inc, USA). The sample solution was injected into the HP6890/ AutoSpec-Ultima equipped with the SCLV Injection System (SGE). Table 1 shows HRGC/HRMS condition.

Results and Discussion
Kitamura showed that dioxins could be measured by using LVI in the serum of 5mL last year.
Referring to Kitamura's method, we tried to establish how to measure dioxins by using SCLV on 10mL of whole blood.
We paid attention to the following point before the examination of the measurement.

1) How to extract a lipid
   The selection of the equipment
   The optimisation of the lipid dry-up condition
2) The verification of the clean-up condition (the adoption of a multiple-layer column connected to an activated carbon silica gel column)
3) The countermeasure of blank level
4) The adoption of SCLV Injection System (SGE)

Figure 1. 80mL centrifuge-tube. Liquid-liquid extraction was done, and a water layer and an organic layer are separated in one centrifuge-tube.

A lipid extraction with centrifuge-tube could make the operation easy (Figure 1).I.S spiked dichloromethane and hexane was dried-up in the weigh-cup 40mm(Figure 2), dichloromethane was dried up in a shorter time in comparison with hexane, and showed a higher recovery ratio. So, dichloromethane 2mL was added to the sample hexane solution 1mL, and a lipid was dried up. A hexane elution volume in a multiple-layer column connected to an activated carbon silica gel column (Figure 3) could obtain best recovery ratio with 25mL (35 mL was needed include washing after loading) in all congeners.
A result is shown in the table 1.It proven that the high sensitivity dioxin analysis is capable with Large volume injection to GC/MS by the adoption of SCLV system.

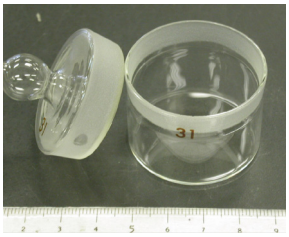

Figure2.
A weigh cup (40mm)
For dry-up a lipid

Because a SCLV system could interrupt a solvent in the middle of the column, the condition of the mass spectrometer can be stabilized for a long time.Measuring lower limit value of dioxins in this way cleared the value that has been required in "2000-year edition blood dioxins measurement temporary manual" of the recommended way in Japan.,and the analysis sensitivity (S/N>5/10fg) was recognized in the blood of 10mL for dioxins analysis, and also good correlation to the method for the blood of 50 mL in temporary manual was recognized.It was recognized that Co-PCB (#118) was contained in the operation blank,though it seems no influence on the measurement value, because it has been detected much more high concentration in the whole blood.As showed in Figure 4,a good correlation of R>0.99 and linearity of almost Y=X had been recognized in the comparison of this method between recommended method.

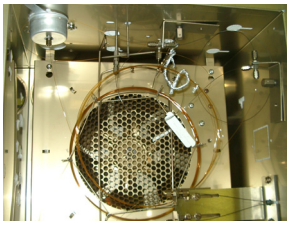

Figure 4.SCLV System

Figure 3.
A multiple-layer column connected to an activated carbon silica gel column

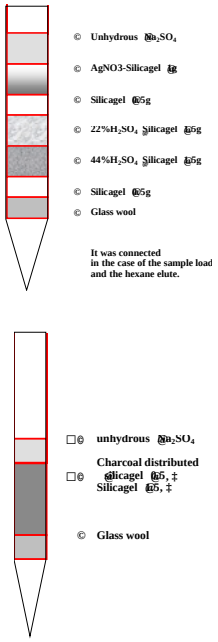

Figure 5.
An example chromatogram of PCDDs , PCDFs , and Co-PCBs in the blood

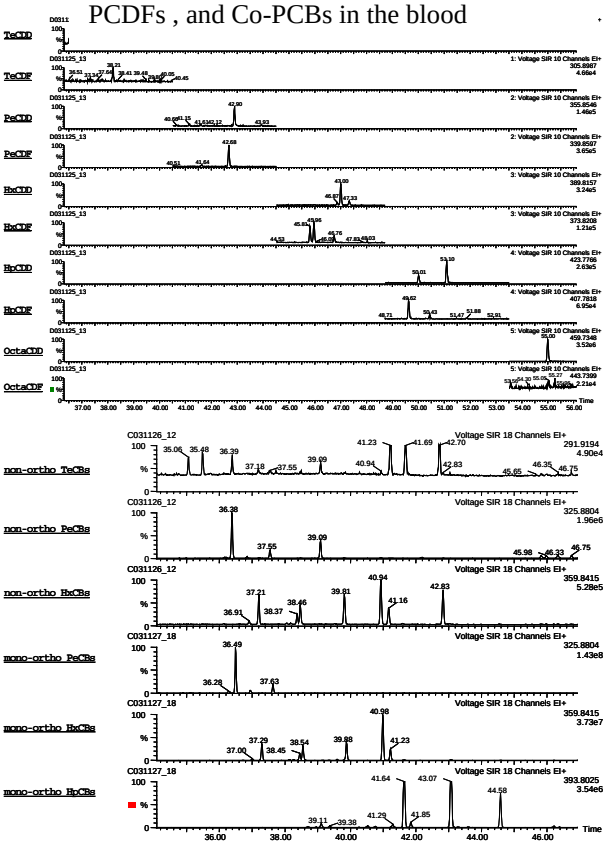

Table 1. The condition of HRGC / HRMS.

GC: 6890N Network GC System (Agilent Technologies inc.) SCLV Injection System (SGE)	
GC Capillary column: BPX-Dioxin-1 (0.15mmID, 30m;SGE)	
Ramp of Oven Temperature 160 °C (5min)-20 °C /min-300 °C (12min)-70 °C /min-195 (0.5min)-3 °C /min –300 °C	

MS AutoSpec-Ultima (micromass)	
Ionizing current: 500μA	Accelerating voltage: 8kV
Ionizing energy: 38eV	Resolution: R >10000 (10% valley)
Measurement Mass: Selected Ion Monitoring (SIM) using PFK	

Dioxins (PCDDs+PCDFs+CoPCBs) analysis method in the blood of 10mLwas established. Measurement lower limit value satisfied the requirement of the way of recommending it. The measurement value of this method showed good correlation in comparison with the recommendation method.

Adoption of the multiple layer silica gel columns connected with the activated carbon silica gel column, an extraction of a lipid by dichloromethane were useful to reduce operation time.
The cleanup by the multiple layer silica gel columns connected with the activated carbon silica gel column, HRGC/HRMS and a Large Volume injection (SCLV) system were effective in the development of the analysis method for dioxins measurement in small volume of blood sample.

Table 2. The comparison between the required method and this method, the coefficient of variation in each congeners measurement value, detection lower limit value.

Inter assay variation (n=5)

@g-TEQ/g-fat j	Reqired method			This method			The ditection lower limit value of the method	All the operation blank value	Reqired measure-ment lower limit value
	Average	S.D.	CV(%)	Average	S.D.	CV(%)	pg/g	pg/g	pg/g
2,3,7,8-TetraCDD	1.5	0.058	3.9	1.8	0.12	6.7	0.0018	0.0012	0.003
1,2,3,7,8-PentaCDD	6.3	0.34	5.4	6.3	0.40	6.3	0.0023	0.0016	0.003
1,2,3,4,7,8-HexaCDD	0.25	0.0053	2.1	0.25	0.016	6.5	0.0022	0.0014	0.006
1,2,3,6,7,8-HexaCDD	2.3	0.068	3.0	2.3	0.12	5.4	0.0017	0.0018	0.006
1,2,3,7,8,9-HexaCDD	0.39	0.010	2.6	0.40	0.031	7.9	0.0017	0.0011	0.006
1,2,3,4,6,7,8-HeptaCDD	0.18	0.0040	2.3	0.19	0.010	5.4	0.0017	0.0026	0.006
OctaCDD	0.040	0.0012	3.0	0.040	0.0010	2.5	0.0024	0.0040	0.01
Total PCDDs	11	0.38	3.4	11	0.34	3.1			
2,3,7,8-TetraCDF	0.090	0.0017	1.9	0.10	0.017	16.7	0.0013	0.00069	0.003
1,2,3,7,8-PentaCDF	0.033	0.0023	7.0	0.044	0.0052	11.6	0.0011	0.00067	0.003
2,3,4,7,8-PentaCDF	6.3	0.30	4.7	6.4	0.21	3.3	0.0019	0.0013	0.003
1,2,3,4,7,8-HexaCDF	0.40	0.022	5.6	0.41	0.019	4.6	0.0010	0.0011	0.005
1,2,3,6,7,8-HexaCDF	0.52	0.018	3.4	0.54	0.034	6.2	0.0016	0.0012	0.005
1,2,3,7,8,9-HexaCDF	|	|	|	|	|	|	0.0019	0.0014	0.005
2,3,4,6,7,8-HexaCDF	0.17	0.0061	3.7	0.19	0.012	6.3	0.0011	0.0011	0.005
1,2,3,4,6,7,8-HeptaCDF	0.028	0.0011	3.9	0.029	0.0010	3.5	0.0014	0.0010	0.005
1,2,3,4,7,8,9-HeptaCDF	|	|	|	|	|	|	0.0018	0.0012	0.005
OctaCDF	|	|	|	|	|	|	0.0025	0.0022	0.01
Total PCDFs	7.5	0.33	4.4	7.7	0.24	3.1			
Total (PCDDs ₱CDFs)	18	0.62	3.4	19	0.38	2.0			
3,3',4,4'-TetraCB(#77)	0.00051	0.000010	2.0	|	|	|	0.0014	0.0039	0.03
3,4,4',5-TetraCB(#81)	0.00025	0.000011	4.3	|	|	|	0.0016	0.00084	0.03
3,3',4,4',5-PentaCB(#126)	7.1	0.22	3.1	7.2	0.26	3.6	0.0015	0.00086	0.03
3,3',4,4',5,5'-HexaCB(#169)	0.42	0.017	4.1	0.42	0.025	5.9	0.0014	0.00095	0.03
Total non-ortho Co-PCBs	7.5	0.23	3.1	7.6	0.28	3.7			
2,3,3',4,4'-PentaCB(#105)	0.27	0.0093	3.4	0.28	0.0067	2.4	0.0023	0.0062	0.03
2,3,4,4',5-PentaCB(#114)	0.42	0.016	3.7	0.43	0.011	2.5	0.0013	0.0013	0.03
2,3'4,4',5-PentaCB(#118)	1.4	0.043	3.1	1.4	0.033	2.3	0.0061	0.0244	0.03
2',3,4,4',5-PentaCB(#123)	0.037	0.0037	10.0	0.036	0.0048	13.4	0.0017	0.0018	0.03
2,3,3',4,4',5-HexaCB(#156)	2.3	0.065	2.9	2.3	0.073	3.2	0.0014	0.0044	0.03
2,3,3',4,4',5'-HexaCB(#157)	0.60	0.017	2.8	0.61	0.019	3.1	0.0014	0.0013	0.03
2,3',4,4',5,5'-HexaCB(#167)	0.019	0.00054	2.9	0.019	0.00036	1.9	0.0023	0.0028	0.03
2,3,3',4,4',5,5'-HeptaCB(#189)	0.048	0.0014	3.0	0.048	0.0011	2.3	0.0020	0.0017	0.03
Total mono-ortho Co-PCBs	5.0	0.15	2.9	5.1	0.14	2.8	-	-	-
Total Co-PCBs	13	0.36	2.9	13	0.41	3.3	-	-	-
Total (PCDDs/PCDFs ₢o-PCBs)	**31**	**0.96**	**3.1**	**32**	**0.74**	**2.3**	-	-	-

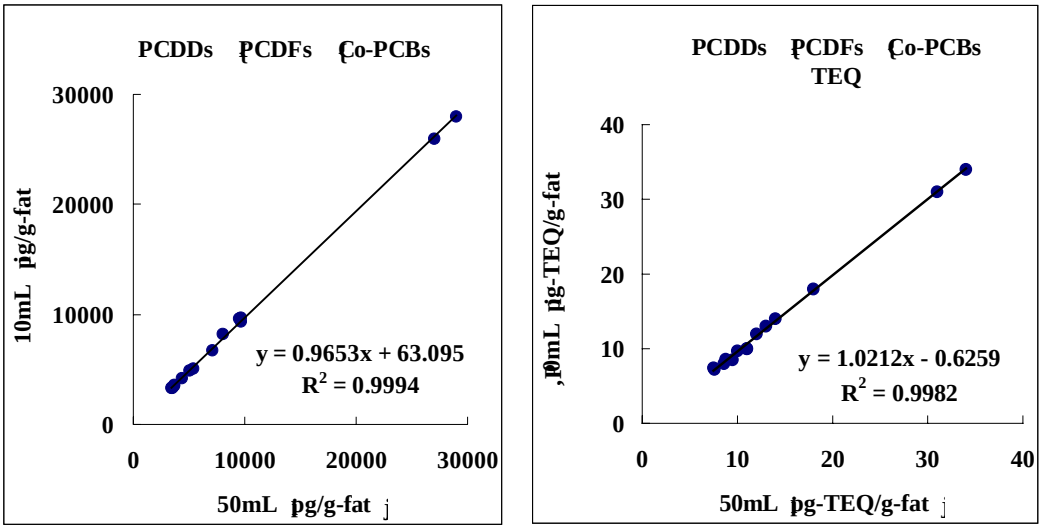

Figure 4. Correlation in the measurement of PCDDs+PCDFs and CoPCB, recommendation method and this method (The left: concentration, the right: TEQ value)

References

1 Kitamura K., Takei K., Choi J., Hashimoto S., Ito H., and Morita M., (2003) Dioxin 2003,60,29.
2 Ray Fischer. et al. (2003) Dioxin 2003,60,21.