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Development of a Newly Large-Volume Injection System for Dioxin Determinations in Blood of Yusho Patients

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Abstract We developed a more effective method to measure the concentrations of polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and non-*ortho*-coplanar polychlorinated biphenyls (non-*ortho* PCBs) in the blood of Yusho patients using high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS) equipped with a newly large-volume injection system. The new injection system linked a LaviStoma system with a unique stomach-shaped inlet liner (SSIL) and a solvent-cut large-volume (SCLV) injection system. This approach made it possible to introduce volumes up to 200μl into the HRGC/HRMS in comparison with the 20μl volume of the previously reported conventional SCLV method. Based on experiments conducted using the same blood sample, the concentrations of PCDDs, PCDFs, and non-*ortho* PCBs obtained by the developed method showed a close correlation to that by the conventional SCLV method. By improving the injection method, the operation time and labor for the purification procedure from blood could be reduced. Furthermore, the developed method was more effective than the conventional SCLV method for recovery of PCDDs, PCDFs, and non-*ortho* PCBs.

Key words : Polychlorinated dibenzo-*p*-dioxins・Polychlorinated dibenzopfurans・Non-*ortho*-coplanar polychlorinated biphenyls・Human blood・Yusho

Introduction

Over 40 years have passed since the outbreak of Yusho disease, and Yusho patients still have much higher concentrations of PCDFs in their blood than do unaffected persons[1)]. Therefore, a follow-up study of PCDDs, PCDFs, and non-*ortho* PCB concentrations in the blood of Yusho patients is very important when considering the health care of these patients. We previously developed an analytical method for accurately determining the concentrations of PCDDs, PCDFs, and non-*ortho* PCBs at a blood volume of 5 g[2)~4)]. Moreover, we measured the concentrations of PCDDs, PCDFs, and non-*ortho* PCBs in the blood collected from Yusho patients during medical health examinations performed from 2002 to 2010[5)~10)]. However, there are still some problems concerning the purification procedures from human blood.

In the purification procedure of PCDDs, PCDFs, and non-*ortho* PCBs from human blood, the toluene eluate (40 ml) obtained by final column chromatography is concentrated to about 200μl with a sample concentrator, and must subsequently be concentrated to 20μl with a solvent evaporation under a nitrogen gas stream. This micro-concentration procedure is very trouble-

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some and time-consuming, and the recovery of PCDDs, PCDFs, and non-*ortho* PCBs may be affected. Therefore, it is necessary to develop a large-volume injection system that can handle volumes up to 200μl of sample solution.

The large-volume injection system using SCLV injectors is based on the selective separation of the solvent from the pre-column, and venting only to the solvent through a solvent-cut valve, while the analytic compounds are focused and condensed in a cold trap component and are separated by the analytic column. Because most interfering matrices in an injected sample can be removed by the pre-column of a SCLV injection system, the analytical column can use a narrow-bore (0.15 mm) column[11]. However, this injection system has the drawback that the volume of injection is limited because the capacity of the straight inlet liner is small.

A LaviStoma system, which employs a large-volume injection procedure using a unique large-capacity SSIL, has been reported to be the most useful for analysis of environmental samples[12]. The large-volume sample (200μl) injected into the SSIL remains in the liquid phase. Then, the solvent is evaporated and the sample is concentrated under a carrier gas stream and the concentrated sample flows into the analytical column. In a LaviStoma system, most of the solvent vapor is excreted via the split vent line. However, the remaining small amount of solvent vapor after a short time of solvent concentration in the SSIL might flow into the analytical column. Therefore, the analytical column cannot be a narrow-bore (0.15 mm) column. Furthermore, a longer period of solvent concentration in the SSIL results in the loss of analytes. The method linked a LaviStoma system and a SCLV injection system is expected to make possible the introduction of sample volumes up to 200μl as compared to the 20μl volume of the conventional SCLV method. Moreover, when the solvent concentration in the SSIL is insufficient, the injected solvent containing the analytes is vented to waste through a solvent cut valve by the SCLV system.

In the present study, we developed a newly large-volume injection technique that linked a LaviStoma system and a SCLV injection system in order to overcome the drawbacks of the two injection techniques described above.

Materials and Methods

1. Materials

Native congeners of PCDDs, PCDFs, and non-*ortho* PCBs were purchased from Kanto Chemical Industries, Ltd., Tokyo, Japan. [$^{13}C_{12}$]-congeners of PCDDs, PCDFs, and non-*ortho* PCBs as internal standards were also purchased from Kanto Chemical Industries, Ltd., Tokyo, Japan. An active carbon column was prepared as follows : active carbon was purchased from Nacalai Tesque (Kyoto, Japan), refluxed 3 times with toluene for 1 hour, and dried in a vacuum, after which 500 mg of the active carbon was mixed with 500 g of anhydrous sodium sulfate (Wako Pure Chemical Industries, Ltd., Tokyo, Japan). A silver nitrate/silica gel was purchased from Wako Pure Chemical Industries, Ltd. All reagents and solvents used in this experiment were of the analytic grade of dioxin that is commercially available.

2. Sample preparation

The extraction and purification of PCDDs, PCDFs, and non-*ortho* PCBs from human blood was performed using a previously reported method[2)~4)].

3. Analysis of PCDDs, PCDFs and Co-PCBs

Concentrations of the PCDDs, PCDFs, and non-*ortho* PCBs were measured using HRGC/HRMS equipped with a LaviStoma system and a SCLV injection system. The analytical conditions were as follows : the gas chromatograph was an HP-7890 A (Agilent Technologies Inc., USA) equipped with a CombiPAL (CTC Analytics AG, Switzerland), an AutoSpec-Premier, (Micromass Ltd., UK), a SCLV injection system (SGE Ltd.,

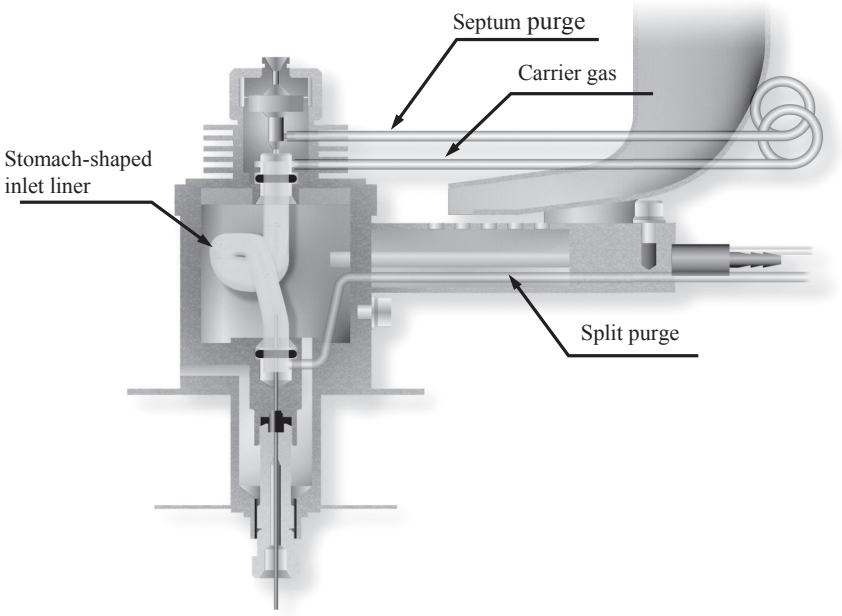

Fig. 1 Inside structure of a LaviStoma system.

Australia) and a LaviStoma system (AiSTI SCIENCE, Japan) (Fig. 1) ; the column used was an BPX-5 fused silica pre-capillary column, 0.25 mm i.d.× 7 m, 0.25μm film thickness (SGE Ltd., Australia) ; the analytical column, 0.15 mm i.d.× 30 m (SGE Ltd., Australia). The injection temperature and ion source temperature were maintained at 120℃ and 280℃, respectively, and the carrier gas (helium) flow rate (constant flow) was 1.3 ml/min. The ionizing energy, accelerating voltage, and trap current were 40 eV, 7.8 kV and 750μA, respectively. PCDDs, PCDFs and non-*ortho* PCBs were analyzed in a single-ion recording mode. The resolution was maintained at 10000 at 10%. PCDDs, PCDFs, and and non-*ortho* PCBs were quantified using one molecular $(M)^+$ ion, $(M + 2)^+$ ion, and $(M + 4)^+$ ion.

Results and discussion

A newly large-volume injection system was developed to overcome the drawbacks of a LaviStoma system and a SCLV injection system. The principle of the method is as follows : In the first stage, the injector is kept at a low temperature, and the large volume of sample injected into the SSIL remains in the liquid phase in the liner. In the second stage, the solvent is evaporated and the sample is concentrated under carrier gas flow. In the third stage, the sample is introduced into an analytical column by raising the temperature of the SSIL in the splitless mode. Finally, in the last stage, the residual solvent or high-boiling-point impurities are purged out in the split mode (Fig. 2). By an SCLV injection system, the solvent and the analytic compound in the samples are separated by the pre-column to remove the various interferences in the sample. The solvent 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 component, and the analytical column is then heated by the temperature program, and the analytic compounds are separated and determined (Fig. 3).

Several parameters for the operation of the LaviStoma system were optimized. The initial injector temperature was set near the solvent boiling point to enable solvent evaporation according to LaviStoma advisable temperature program. Because the solvent used in the analysis was toluene and its boiling point is 110℃, the initial injector temperature was set at 120℃. The analytical conditions were as follows : the injection

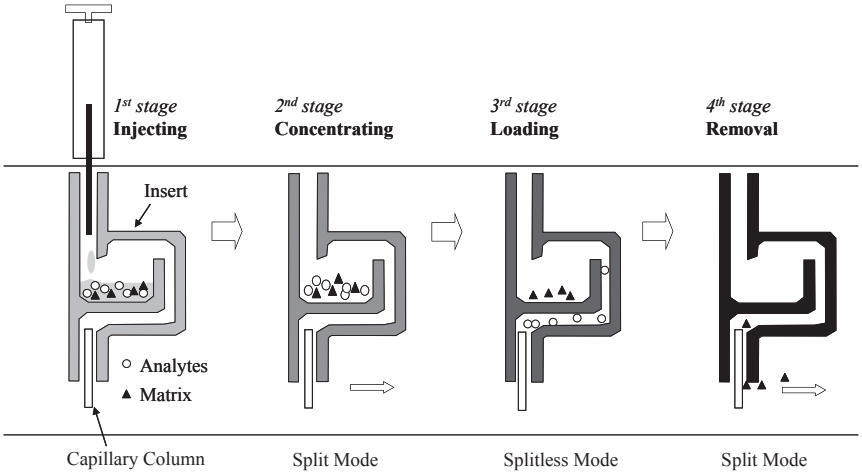

Fig. 2 Principle of injection method with a LaviStoma system.

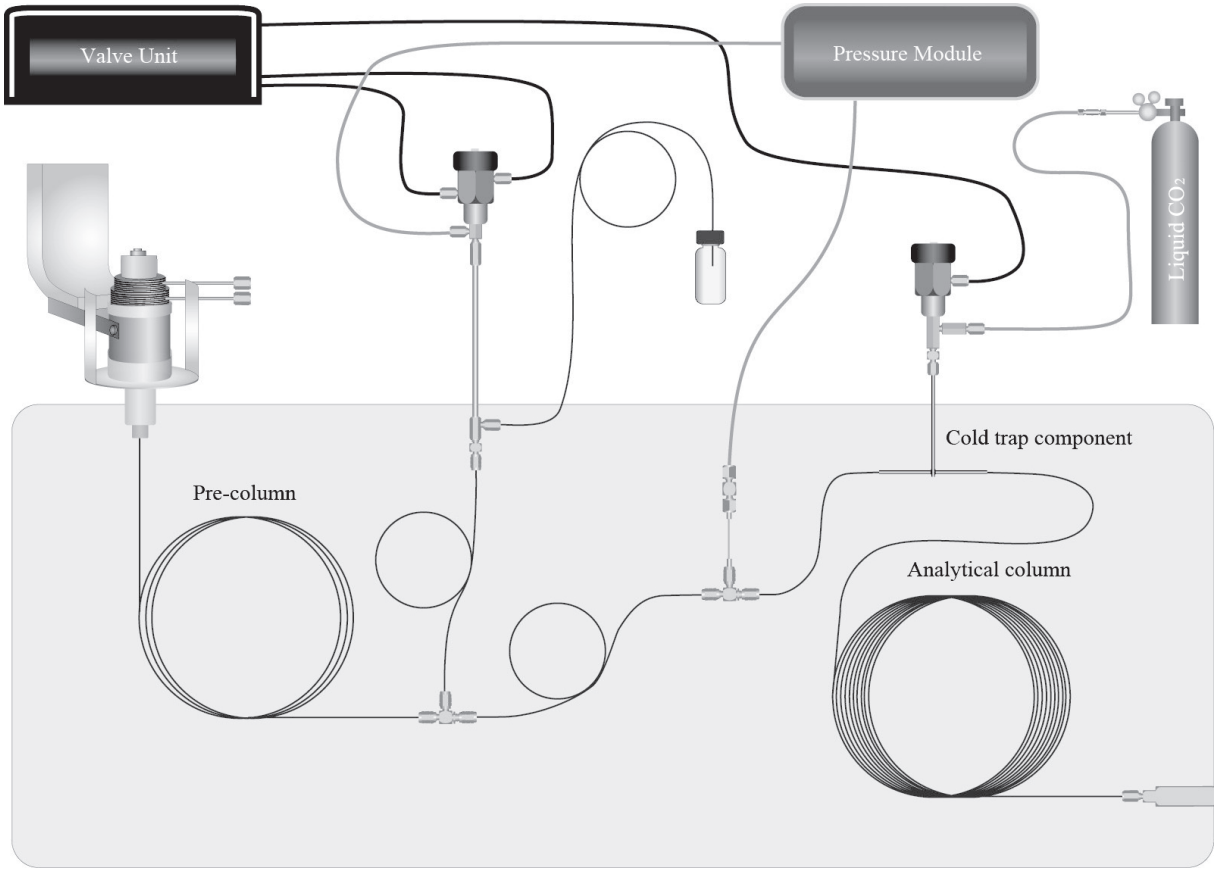

Fig. 3 Principle of a LaviStoma-SCLV system developed in the present study.

temperature was maintained at 120℃ for 1 min, heated from 120℃ to 290℃ at a rate of 120℃ /min, and maintained at 290℃ for 20.3 min ; meanwhile, the column oven temperature was maintained at 160℃ for 3.75 min, heated from 160℃ to 300℃ at a rate of 20℃ /min, maintained at 300℃ for 12 min, cooled to 195℃ at a rate of 70℃ /min, maintained at 195℃ for 0.5 min, heated to 300℃ at a rate of 3℃ /min, and then maintained at 300℃ for 1.75 min.

Because the injection volume, the purge time, the vent flow rate, and the vent time are critical factors for the operation of a LaviStoma-SCLV system, the relationships between these parameters were examined at different volumes ; thus, 25, 50, 100, and 200μl of the standard solutions (0.01 ng ml^{-1}) of PCDDs, PCDFs, and non-*ortho* PCBs were injected using a LaviStoma-SCLV system under the operating conditions mentioned above. The optimization was conducted by comparing the peak areas of individual congeners of these compounds for a LaviStoma-SCLV system with those for the conventional SCLV system. The optimization purge times for injection of the standard solution volumes of 25, 50, 100, and 200μl were 3.75, 3.75, 3.75, and 4.75 min, respectively, and the vent flow rates were 150, 300, 300, and 300 ml/min, respectively, with vent times of 0.1, 0.5, 1.0, and 2.0 min, respectively. Under these conditions, the relationship between the peak area of an individual congener and the injected volumes (25, 50, 100, and 200μl) of the standard solution of PCDDs, PCDFs, and non-*ortho* PCBs was examined. When each volume of the standard solution was injected 3 times with a LaviStoma-SCLV system, the calibration curves of individual congeners of these compounds all showed good linearity ($R > 0.99$) in the range of 25-200μl, and the relative standard deviations of three replicate determinations for different injection volume of the standard solution were all below 20%, indicating good reproducibility.

We performed a preliminary study regarding the calibration curves of the individual congeners of PCDDs, PCDFs, and non-*ortho* PCBs with a LaviStoma-SCLV system. Calibration standard solutions ranging from 0.5 to 50 pg ml^{-1} were prepared by diluting the standard solution (0.25 ng ml^{-1}) of PCDDs, PCDFs, and non-*ortho* PCBs, and volumes of 100μl were injected into the LaviStoma-SCLV system. The calibration curves of individual congeners of these compounds all showed good linearity ($R > 0.99$), and the relative standard deviation at each dose was less than 20%.

The concentrations of PCDDs, PCDFs, and non-*ortho* PCBs measured by the developed method and the previously reported method using the conventional SCLV method were compared for blood samples collected from 22 normal subjects. The concentrations of individual congeners of PCDDs, PCDFs, and non-*ortho* PCBs prepared by both methods were nearly the same, and the total toxicity equivalency factor (TEQ) levels were almost equal to those obtained by the conventional SCLV method. These findings indicate that the developed method is essentially equivalent to the conventional SCLV method. However, recovery of the ^{13}C-labeled internal standard was increased about 20% overall compared with the conventional SCLV method. In addition, the developed method demonstrated high reproducibility based on experiments conducted using the same control serum sample for 12 weeks (Table 1). These data indicated that the developed method is applicable for the determination of PCDDs, PCDFs, and non-*ortho* PCBs in the blood of Yusho patients.

In the previously reported method, the dioxin fractions (40 ml) eluted with toluene for final purification procedure from human blood were concentrated to about 200μl with a sample concentrator. Moreover, the sample had to be concentrated to 20μl with solvent evaporation under a nitrogen gas stream. This procedure is very troublesome and time-consuming, and the recovery of PCDDs, PCDFs, and non-*ortho* PCBs

Table 1 Reproducibility test of the developed method conducted using the same control serum sample for 12weeks

Congeners	Concentration (pg g^{-1} lipid)											
	Week											
	1	2	3	4	5	6	7	8	9	10	11	12
2,3,7,8-TetraCDD	1.6	1.7	1.4	1.3	1.9	2.0	1.4	1.2	1.2	1.4	1.7	1.5
1,2,3,7,8-PentaCDD	5.2	6.1	6.2	6.5	7.2	6.4	6.1	6.4	6.8	7.0	6.8	5.4
1,2,3,4,7,8-HexaCDD	5.3	7.0	5.9	5.6	5.6	3.6	6.9	7.0	7.5	5.7	5.6	11.0
1,2,3,6,7,8-HexaCDD	50	51	42	44	44	47	47	45	41	43	44	44
1,2,3,7,8,9-HexaCDD	11	9.1	7.1	8.7	6.7	5.7	8.5	8.3	8.0	7.0	7.9	7.0
1,2,3,4,6,7,8-HeptaCDD	145	178	119	113	105	96	112	144	162	133	120	121
OctaCDD	1427	1432	1379	1453	1017	1046	1546	1480	1301	1187	1195	1420
2,3,7,8-TetraCDF	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
1,2,3,7,8-PentaCDF	1.4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2,3,4,7,8-PentaCDF	6.6	4.8	6.0	5.4	5.2	5.6	6.3	5.3	5.3	5.0	4.7	5.8
1,2,3,4,7,8-HexaCDF	7.7	5.7	6.2	6.7	5.8	6.2	6.1	5.5	4.4	5.5	5.5	5.1
1,2,3,6,7,8-HexaCDF	6.0	6.1	5.9	6.5	5.3	5.6	5.7	5.9	5.5	5.0	4.9	6.6
2,3,4,6,7,8-HexaCDF	ND	ND	ND	ND	ND	2.1	ND	ND	ND	ND	ND	ND
1,2,3,7,8,9-HexaCDF	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
1,2,3,4,6,7,8-HeptaCDF	14	13	15	15	14	12	15	14	13	11	14	14
1,2,3,4,7,8,9-HeptaCDF	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
OctaCDF	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
TriCB-77	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
TriCB-81	43	38	40	39	37	41	41	38	36	35	37	43
PentaCB-126	23	20	24	23	21	23	25	25	25	21	22	25
HexaCB-169	19	18	22	19	19	19	19	19	19	16	18	19
Total PCDD	1645	1684	1561	1633	1188	1207	1728	1692	1527	1383	1382	1610
Total PCDF	41	35	40	40	36	42	40	37	34	33	35	38
Total PCDD/PCDF	1686	1719	1601	1673	1224	1249	1767	1729	1560	1416	1417	1648
Total Non-ortho PCBs	90	81	92	86	82	88	89	88	84	77	82	92
Total	1776	1800	1692	1759	1305	1336	1856	1817	1644	1493	1499	1740
TEQ from PCDDs	15	17	15	15	16	15	15	16	16	16	16	15
TEQ from PCDFs	3.8	3.0	3.5	3.4	3.1	3.9	3.5	3.2	3.0	2.9	2.9	3.3
TEQ from PCDDs/PCDFs	19	20	18	19	19	19	19	19	19	19	19	18
TEQ from non-*ortho* PCBs	2.9	2.6	3.1	2.9	2.7	2.9	3.0	3.1	3.0	2.6	2.7	3.1
Total TEQ	22.0	22.3	21.2	21.5	21.8	22.0	21.9	21.9	21.7	21.2	21.4	21.2
Lipid (%)	0.31	0.31	0.28	0.29	0.33	0.27	0.27	0.27	0.30	0.29	0.29	0.28

ND : less than the determination limit.
CDD : chlorinated dibenzo-*p*-dioxin.
CDF : chlorinated dibenzofuran.
CB : chlorinated biphenyl.

may be affected. The developed method made it possible to introduce volumes up to 200μl into the system. Consequently, the micro-concentration procedure by a solvent evaporation under nitrogen gas stream could be excluded, and it was possible to reduce the time and labor involved, and lessen the danger of the escape of PCDDs, PCDFs, and non-*ortho* PCBs. These findings indicate that the developed method was more effective than the previously reported method for efficiency of operation procedure and determination of PCDDs, PCDFs, and non-*ortho* PCBs.

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（和文抄録）

油症患者血液中ダイオキシン類分析における新しい大量注入法の検討

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油症患者血液中 PCDDs，PCDFs および non-*ortho* PCBs 分析を新しい大量注入装置を装備した高分解能ガスクロマトグラフ/高分解能質量分析法（HRGC/HRMS）を用いて検討した．新しい大量注入装置は胃袋型インサートを備えた LaviStoma システムと Solvent-cut large-volume injection（SCLV）システムを連結して用いた．この装置を用いて，注入条件を検討した結果，200μl の試料注入が可能となった．同一の血液を用いて行った従来の SCLV 法との比較検討の結果，本法は従来法と同様な結果が得られることが確認された．本法は従来の SCLV 法に比べ，血液試料の前処理段階で費やす時間を短縮することができ，PCDDs，PCDFs および non-*ortho* PCBs の回収率も約 20％向上した．