DEVELOPMENT OF SIMPLE AND RAPID PURIFICATION METHODS FOR BIOANALYTICAL DETECTION OF DIOXINS

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Introduction

Polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) are organochlorinated pollutants found in various environmental matrices. These compounds are known to be bioaccumulative exhibiting a variety of toxic effects including tumor promotion, immunotoxicity, reproductive toxicity and endocrine disruption. In consequence PCDD/Fs are recognized as a potential threat to human health and to be a serious ecological issue.

High-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS) has conventionally been used for detection of PCDD/Fs because of the advantages of separating the numerous congeners and simultaneous quantification of each component with reasonable accuracy and precision. However, the HRGC/HRMS procedures require time-consuming and expensive pretreatment method to extract a trace amount of the target compounds.

Thus, several rapid and high-throughput bioanalytical methods, involving enzyme-linked immunosorbent assay (ELISA)[1,2] and AhR-dependent assay[3-5], have recently been developed. These procedures could be much more feasible, cheaper and suitable for routine monitoring of potential toxicity due to PCDD/Fs in various samples with a reasonably low detection limit. However, a proper sample pretreatment is still necessary to avoid interference particularly due to lipophilic substance: that masks the target compounds and prohibit to be bound by antibody or receptor.

In this study, we established simple, rapid and inexpensive purification procedures for an ELISA of PCDD/Fs using a solid phase extraction (SPE) cartridge, Wakogel P-29. Moreover, we attempted to develop an immunoaffinity extraction method that could allow even simpler and faster pretreatment for bioanalyses of PCDD/Fs. To achieve this aim, previously generated monoclonal antibodies, whose cross-reactivity to dioxin congeners was corresponding to the toxic equivalence factors (TEF), were employed for preparing immunosorbent.

Materials and Methods

Chemicals

PCDD/Fs congeners were purchased from Wellington Laboratories. SPE cartridges, Wakogel P-29 and abselut NEXUS, were purchased from Wako Pure Chemicals and Varian, respectively. Cyanogen

bromide (CNBr)-activated Sepharose 4FF was obtained from Amersham Pharmacia Biotech, and Affi-Gel 10 was from Bio-Lad.

Clean-up Procedure for ELISA

Fat was saponified with 1 mol/L KOH and extracted with *n*-hexane. The organic layer was washed with H_2SO_4 until the sulfuric acid layer had become to be clear. The extract was loaded to Wakogel P-29 cartridges, which were washed with *n*-hexane and then eluted with hexane/benzene (3:1). After addition of Triton X-100 (0.1% in MeOH; 25µL) to the effluent, the solvent was evaporated and the residue was dissolved in PBS, then the following ELISA performed.

ELISA

The ELISA was carried out using monoclonal antibody D9-36 as described previously[2]. Briefly, horseradish peroxidase-labeled hapten, the monoclonal antibody and standard dioxin or the sample prepared as above were added to the second antibody-coated wells in a 96-well microtiter plate. After overnight incubation at 4 ºC, the wells were washed with PBS, and the bound enzyme activity was measured using H_2O_2 and *o*-phenylenediamine as a substrate.

Preparation of Immunosorbent and Affinity Column

A solution of the IgG fraction (monoclonal antibody D2-37, D9-36 or D35-42)[2] was added to the CNBr-activated Sepharose 4FF or Affi-Gel 10. After gentle stirring of the mixture overnight at 4?, remaining reactive groups of the gel were blocked by addition of 0.1 mol/L Tris-HCl buffer (pH 8.0). Then the gel was washed serially with 0.1 mol/L acetate buffer (pH 4.0), 95% MeOH, water and PBS, and a portion of the resulting gel (1 mL) was packed into a disposable column.

Immunoaffinity Extraction-Based Pretreatment

Fat specimen was treated as described above, and the residue was dissolved in PBS and applied to the immunoaffinity column. After washing serially with PBS, water and 10% MeOH, PCDD/Fs were eluted with 95 % MeOH. This fraction was diluted with water to reduce the MeOH concentration to be less than 60 %, added to the NEXUS cartridge and washed with 90% MeOH. Then PCDD/Fs fraction was eluted with acetone, and submitted to the ELISA.

GC/MS Measurement

After purification by Wakogel P-29, PCDD/Fs in the purified extract were determined by HRGC/HRMS on a Hewlett Packard 5890-II gas chromatograph-JEOL JMS-700 (Mstation) mass spectrometer at a resolution of R=10000 in the selected ion monitoring mode (SIM).

The HRGC/HRMS conditions were as follows: column, Supelco 2331 60 m x 0.25 mm i.d., 0.25µm thickness; column temperature program, 130º (2 min), 130-200º at 15º/min, 200-260º at 3?/min, 260 ºC (30 min); carrier gas, He; column head pressure, 168 Kpa; injection temperature, 270º; injection volume, 2 µL (splitless). The MS conditions: detection mode, EI; ion source temperature, 270 ; ionizing current, 600 µA; ionizing energy, 38 eV; accelerating voltage, 10 KV. The results were corrected for the recovery of $^{13}C_{12}$-labeled internal standards.

Results and Discussion

Saponification of the fat with KOH and washing with H_2SO_4 were essential for developing clean-up procedures of dioxins. After the purification of the extract using Wakogel P-29 cartridge, we could obtain reasonable assay values for PCDD/Fs using the ELISA, which were in good correlation with the values obtained with the GC/MS procedure (fig.1). Recoveries of 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD and

2,3,4,7,8-PeCDF through the clean-up procedure (determined by the GC/MS) were 64 %, 75 % and 85 %, respectively. In the case of the immunoaffinity extraction-based pretreatment, the recovery of 2,3,7,8-TCDD from fat sample (measured by the ELISA) was about 70 %.

Conclusion

It has been shown that the present clean-up procedure using Wakogel P-29 is useful for developing an ELISA system which is available as a simple, rapid and inexpensive method for screening and monitoring of PCDD/Fs. The immunoaffinity extraction using monoclonal antibodies equipping high affinity to toxic dioxin congeners is expected to be a novel pretreatment procedure that is available not only for ELISAs but also for the AhR-dependent assays.

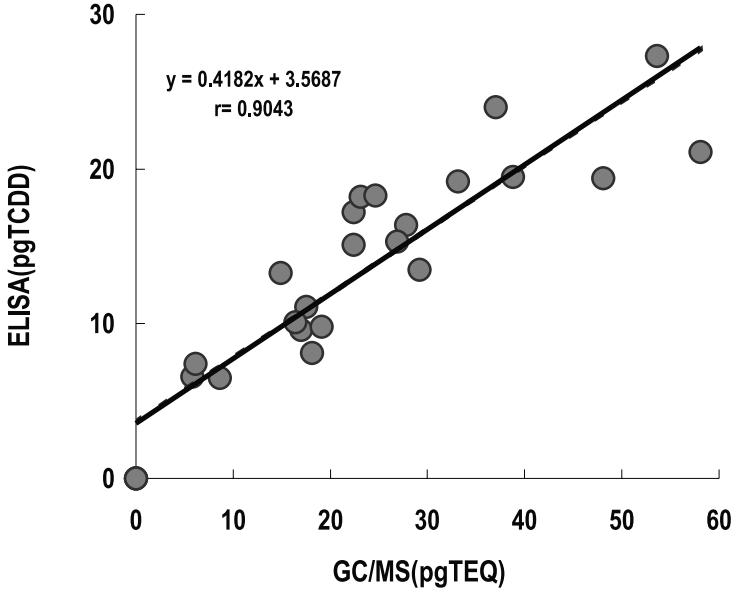

Fig.1 Correlation between GC/MS and ELISA for Fat Samples

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