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Cleanup Method Using Disposable Tandem Cartridge System for the Determination of Dioxins in Human Milk by Enzyme-Linked Immunosorbent Assay

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For dioxin analysis, the establishment of the appropriate analytical method and the quality control procedure are indispensable for reliable measurements. On the other hand, a method that is simple, rapid, inexpensive, and highly sensitive for the screening of dioxins has been highly demanded by the public and the government. One of the methods that may satisfy these requirements is an enzyme-linked immunosorbent assay (ELISA), and there have been some reports on the measurement of dioxins using ELISA. We have already reported the development of a dioxin toxicity evaluation method for human milk by ELISA (Sugawara et al. 1998; Ishizuka et al. 2001; Sugawara et al. 2002; Saito et al. 2003a). However, the dioxin analysis by ELISA usually requires a sufficient cleanup similar to the GC/MS method due to the extremely low content of the dioxins, though ELISA is generally said to have the ability of high specificity.

A cleanup method for the analysis of human milk, in which an alkaline digestion step and a three-layer H_2SO_4/silica-gel cartridge were used, was reported in a previous paper (Saito et al. 2003a). Although an excellent cleanup effect could be obtained by employing this method, it required an overnight alkaline digestion treatment (about 12 hours) and a liquid-liquid distribution extraction using hexane. Accordingly, a more effective cleanup method was developed using the tandem column combined with a commercially available multi-layer silica-gel cartridge and an alumina cartridge. Furthermore, in order to assess the usefulness of the proposed cleanup method, the assay validation was carried out by comparing a conventional GC/MS method and the proposed ELISA method for the determination of dioxins in human milk samples.

MATERIALS AND METHODS

All of the dioxin standards were from Wellington Laboratories (USA) and were diluted with decane to the appropriate concentrations. Most of the organic solvents such as hexane, acetone, dichloromethane, toluene, diethylether, ethanol and methanol (MeOH) were of dioxin analysis quality from Kanto Kagaku (Tokyo, Japan) or Wako Pure Chemicals (Osaka, Japan). Decane was of special quality

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grade and was redistilled prior to use.

The multi-layer silica-gel column packed in a disposable cartridge tube was from GL Sciences, Inc. (Tokyo, Japan). It is made of 0.9g silica-gel, 3g 2% KOH/silica-gel, 0.9g silica-gel, 4.5g 44% H_2SO_4/silica-gel, 6g 22% H_2SO_4/silica-gel, 0.9g silica-gel, 3g 10% $AgNO_3$/silica-gel and 6g sodium sulfate. The cartridge was washed with 100 mL of hexane prior to use. The Sep-pak Plus Alumina (Basic type) cartridge was from Waters (Japan), and was washed with 10 mL of hexane prior to use. The tandem cartridge column of a multi-layer silica-gel cartridge and a Sep-pak Plus Alumina cartridge was prepared as follows; the column outlet part of the multi-layer silica-gel cartridge was connected in series to the inlet part of the Sep-pak Plus Alumina cartridge.

The surrogate standard for ELISA, 2,3,7-trichloro-8-methyldibenzo-*p*-dioxin (TMDD) was synthesized by Sanborn et al. (Sanborn et al.1998). Goat anti-rabbit antibody coupled to horseradish peroxydase and 3,3', 5,5'-tetra-methylbenzidine (TMB) were purchased from Sigma-Aldrich (USA). All other immunoreagents including coating hapten III for 2,3,7,8-TCDD and the antiserum for this ELISA were described in a previous report (Sugawara et al. 2002). All the other chemicals were of PCB analysis quality grade or special quality grade, and used without further purification.

The fat was extracted from human milk according to a previously described procedure (Saito et al. 2003a), and then dissolved with ca.2 mL of hexane. The hexane solution was applied to the tandem cartridge. After washing the cartridge with 160 mL of hexane, the multi-layer silica-gel cartridge, the upper cartridge of the tandem cartridge, was removed, and subsequently the dioxin fraction was obtained by eluting the Sep-pak Plus Alumina cartridge with 5 mL of 60% DCM/hexane. To the eluate was added 60 μL of a 100 ppm Triton X-100 methanol solution as a keeper, the solution was nearly dried out by a nitrogen stream, and the residue was re-dissolved into 60 μL of MeOH-DMSO (1:1).

The ELISA assay was carried out using a dry plate, which was prepared according to the reported method (Ishizuka et al. 2001). An outline of the method is shown below. Briefly, microtiter plates (Sumitomo Bakelite, Tokyo, Japan) were coated with the optimized concentration (0.5 μg/mL, 100 μL/well) of coating antigens (hapten III) (Sugawara et al. 1998) in carbonate-bicarbonate coating buffer (pH 9.6). They were incubated overnight at 4 °C. On the following day, the coated plates were washed 5 times with 0.05% (v/v) Tween 20 in phosphate buffered saline (PBS, pH 7.5) and were incubated for 30 min at room temperature with 300 μL of a 0.5% (w/v) bovine serum albumin (BSA) with sucrose in PBS (blocking solution) per well. After the removal of the blocking solution, the plates were dried *in vacuo* for 4 hr at 25 °C. They were then put into separate aluminum bags, and were packed *in vacuo*. Standards were prepared in 1:1:2 (v:v:v) DMSO:MeOH with 100 ppm Triton X-100 : PBS containing 2 mg/mL BSA

(PBSB). After an initial blocking step with BSA-PBS, and a wash step, 50 μL of the standards were added to the standard wells in a microtiter plate. The sample wells contained 25 μL of PBSB, then 25 μL of a human milk sample in DMSO-MeOH was added. Next, 50 μL of the antiserum diluted in PBSB was added to each well. The final ratio of DMSO-MeOH to PBSB was 1:3. The plates were incubated for 90 min. Following a wash step, 100 μL of goat anti-rabbit antibody coupled to horseradish peroxydase was added (diluted in PBS + 0.05% Tween 20). After a 60-min incubation period, the plates were washed with wash buffer, and 100 μL of an enzyme substrate containing TMB was added to each well. After 20 min, the color reaction was stopped by the addition of 50 μL of 2 M sulfuric acid. The resultant color was measured at 450 nm with a Model 550 Microplate Reader (Bio-Rad Laboratories Inc., Hercules, CA) in the single wavelength mode, and the dioxin levels in the human milk samples were calculated on the basis of a standard curve derived from a fit of absorbance versus the logarithm of the concentration.

The analysis of human milk by the GC/MS method was carried in accordance with a previous report (Saito et al. 2003b). The toxic equivalent quantity (TEQ) was calculated using WHO-TEF1998.

RESULTS AND DISCUSSION

The multi-layer silica-gel cartridge was made by modifying the multi-layer silica-gel column (Lamparski et al. 1980; Smith et al. 1984), which has been widely used for dioxin cleanup in various kinds of samples. A cleanup method for the GC/MS analysis of house dust, in which the usefulness of a multi-layer silica-gel cartridge method compared to an alkaline digestion method, was already reported in a previous paper (Saito et al. 2003b). In our preliminary experiment, we tried to develop a simpler cleanup method that allows the extracted fat to be directly treated using a multi-layer silica-gel cartridge without subjecting it to the alkaline digestion step. The multi-layer silica-gel cartridge was a useful method that was able to effectively remove the coexisting material of matrix origin such as the lipids and pigments in the human milk fat. However, the percentage of the fat content in the human milk depends on the subject. An excess amount of fat was often observed to spoil the cleanup performance of the multi-layer silica-gel cartridge. Moreover, the ELISA occasionally showed a false negative value or a low measurement value due to insufficient purification, even if the level of the fat content was in the range of the processing performance of the multi-layer silica-gel cartridge for the purification. We then tried further purification using alumina column chromatography, which has been also widely used for the dioxin cleanup. We have adopted the idea and created a tandem cartridge column system, i.e., a disposable alumina cartridge connected in series to the output of the multi-layer silica-gel cartridge was prepared in order to improve the cleanup efficiency. Furthermore, the maximum loading level of the human milk fat onto the multi-layer silica-gel cartridge was examined.

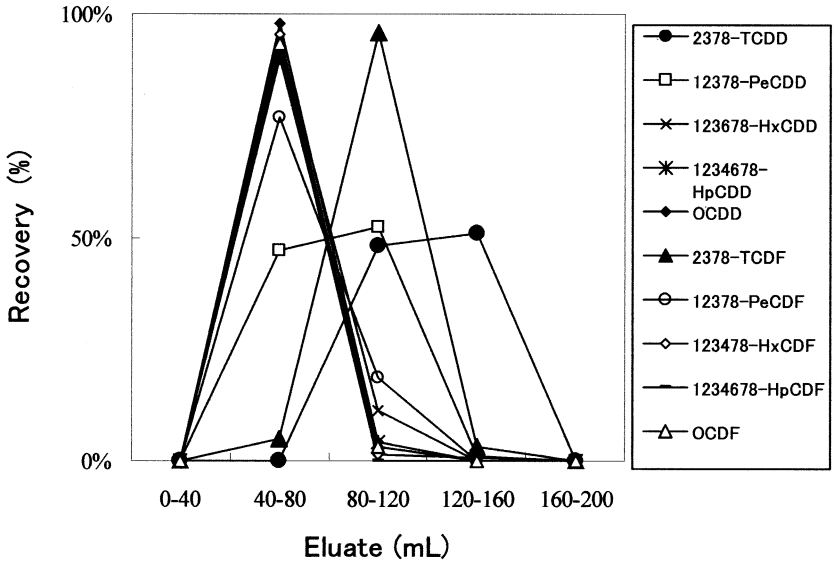

Figure 1. Elution profile of PCDD/Fs from a multi-layer silica-gel cartridge. Forty mL each of hexane eluate was collected as a fraction.

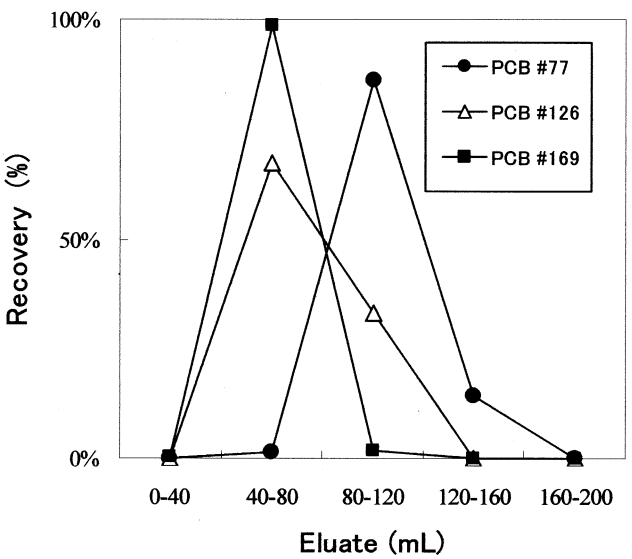

Figure 2. Elution profile of non-ortho PCBs from a multi-layer silica-gel cartridge. Forty mL each of hexane eluate was collected as a fraction.

The maximum loading level of the human milk fat onto the multi-layer silica-gel cartridge was estimated. Each fat (1,1g, 1,2g, 1.3g, 1.5g, 1.8g, 2.3g) extracted from actual human milk (six specimens) was applied to the multi-layer silica-gel cartridge, and the maximum loading level of the fat was judged by observing the carbonization reaction in the 44% H_2SO_4/silica-gel layer of the cartridge. That is, the limit state was that the brown color layer formed along with the reaction in the H_2SO_4/silica-gel layer does not reach the KOH/silica-gel layer. As a result, the fat level of 1.5 g or less was found to be suitable. Accordingly, the fat maximum loading level in the multi-layer silica-gel cartridge was judged to be 1.5g.

The eluting fraction profile of each congener of the PCDD/Fs and Co-PCBs was examined using a multi-layer silica-gel cartridge with hexane as the mobile phase. The dioxin standards, 10 kinds of PCDD/Fs (200 pg each), and 3 kinds of non-ortho-PCBs (1000 pg each) were added to the multi-layer silica-gel cartridge, and subsequently the cartridge was washed with hexane. Each 40 mL of effluent was collected as a separate fraction. Figure 1 and Figure 2 revealed that all of the target chemical substances were eluted with 160 mL of hexane.

For the ELISA, it was preferable to remove the non-planar PCBs and mono-ortho PCBs eluted from the multi-layer silica-gel cartridge as much as possible. There were a lot of PCBs residues in the eluate although the cross-reactivity of these PCBs in the ELISA is extremely low compared to the PCDD/Fs. We then paid attention to the alumina column chromatography already used in the analysis of dioxin, and examined a simple cleanup method with a commercially available pre-packed alumina cartridge. In that case, in order to simplify the operation as much as possible, the alumina cartridge was connected under the multi-layer silica-gel cartridge in series. Such an operation was based on the following idea: dioxins seemed not to be easily eluted from the alumina cartridge when using a non-polar solvent such as hexane, because the basic alumina has the ability to maintain dioxins stronger than silica-gel. The following method was then examined: After the dioxins eluted from the multi-layer silica-gel cartridge were trapped in the alumina cartridge, the multi-layer silica-gel cartridge was removed. Subsequently, the dioxins were eluted from the alumina cartridge with an appropriate solvent. The elution behavior of the dioxins from the alumina cartridge was then examined. As a result, it turned out that both the PCDD/Fs and non-ortho Co-PCBs were not eluted in the first 160mL of hexane, and were eluted in a subsequent 5mL of 60% DCM/hexane.

Figure 3 shows the relationship between the TMDD equivalents by ELISA and GC/MS values (Total-TEQ) determined in the actual human milk samples. Fairly good agreements between the GC/MS values and ELISA values were obtained from a linear regression analysis ($r = 0.947$, $n = 7$). The strong correlation between ELISA and TEQ suggests that the ELISA using the proposed cleanup method indicated its usefulness as a toxicity evaluation method for dioxins in human milk.

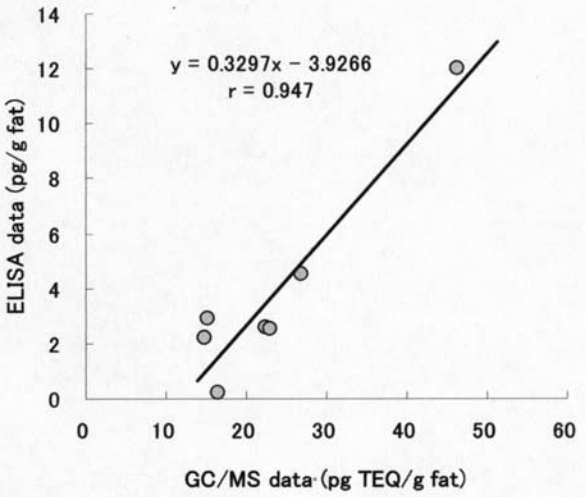

Figure 3. Correlation between GC/MS data and ELISA data.

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