Development of Dioxin Toxicity Evaluation Method in Human Milk by Enzyme-Linked Immunosorbent Assay (part II - Examination of Preprocessing Technique to Make ELISA compatible with GC/MS Method)

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Introduction

High-resolution gas chromatography/mass spectrometry (HR-GC/MS) is being used for the measurement of dioxin. However, the GC/MS method has drawbacks in that it requires a complicated cleanup procedure for the measurement of all types of samples and that it is very time consuming and extremely expensive to perform. Thus, an enzyme linked immunosorbent assay (ELISA) is being examined as one of the inexpensive and easier methods of measuring dioxins with high sensitivity. There have already been some reports using ELISA. However, most of these reports were intended for standard substances[1,2], fly ash[3], the soil[4,5], and chimney soot[6], which were contaminated by dioxins in high concentration. There has been no report on a practicable analysis method intended for biological samples containing dioxins in extremely low levels, such as human milk and blood.

Sugawara, who is our co-investigator, has already reported an ELISA for dioxin measurement with high sensitivity[1]. In addition, we examined the utility as a toxicity evaluation method to which ELISA was used as not an only handy screening method but a method by which the toxicity could be immediately evaluated intended for the biological samples. These results will be described in other presentations (parts I and III) at this symposium. In this report, the common preprocessing operation was made compatible with the conventional GC/MS method. Moreover, simplifying a conventional preprocessing operation method that was extremely complicated was examined. We made a prototype of a three-layer sulfuric acid silica gel cartridge that took the place of the multi-layer silica gel column, and also examined its utility for practical use.

Experimental Methods

1. Determination of dioxins in human milk by GC/MS method

According to the conventional method, a stable isotope of one kind or more in each congener of the PCDDs and PCDFs, and $^{13}C_{12}$-1,2,3,4-TCDF were added as a surrogate after the fat was extracted from the human milk. The fat was then subjected to concentrated sulfuric acid washing and then to various chromatographies (silica gel, alumina, and activated carbon silica gel) as the cleanup operation, followed by the GC/MS measurement. The PCDD/Fs were analyzed by HR-

GC/MS using a JEOL JMS-700 mass spectrometer equipped with a capillary DB-17HT column (30 m x 0.25 mm i.d., film thickness 0.15 um). Quantification of the PCDD/Fs were based on fat basis analyses of the following two cases; 1) one kind of stable isotope in each congener of the PCDD/Fs was used for the internal standard for the isotope dilution method, 2) $^{13}C_{12}$-1,2,3,4-TCDF was for the tetra to hexa-CDD/Fs, and $^{13}C_{12}$-OCDD was for the hepta to octa-CDD/Fs. The toxic equivalent quantity (TEQ) was calculated using WHO-TEF (1998).

2. Simplifying of the preprocessing operation

Similar to the manner described above, the fat was extracted from human milk, and surrogates were added to the fat, then 2N KOH/EtOH was added. It was then allowed to stand at room temperature over night. Thus, the alkali decomposition was carried out. The alkaline solution was diluted with water, followed by liquid-liquid extraction with hexane. After the hexane layer was dehydrated and concentrated, the extracted material was processed using a three-layer sulfuric acid silica gel column (silica gel 0.5 g, 44% sulfuric acid silica gel 2 g, and silica gel 1 g are filled to a glass column of 1 cm in the inside diameter), followed by activated carbon silica gel column chromatography. The prototype of three-layer sulfuric acid silica gel column packed in a disposable cartridge tube (inside diameter 15 mm x length 75 mm; made of polypropylene) was made by special request to Supelco.

Results and Discussion

1. Examination of surrogates to make ELISA compatible with GC/MS method

As the surrogates usually used for the conventional GC/MS method are 2,3,7,8-substituted $^{13}C_{12}$-congeners and they react with the antibody used for ELISA, the test solution prepared for a GC/MS method cannot be applied to ELISA. Therefore, we decided to examine the kinds of surrogates that do not cause a cross reaction with ELISA, and that the measurement of the isomers is possible with good accuracy by the GC/MS method in this research. As for the antibody that had been used by this research, there was a tendency that it strongly reacted with the congeners of high TEF[1]. Therefore, we selected $^{13}C_{12}$-1,2,3,4-TCDF for the 4-6CDD/Fs measurement and $^{13}C_{12}$-OCDD for the 7,8CDD/Fs measurement as surrogates that meet the above-mentioned requirement. The TEFs of these two congeners are 0 and 0.0001, respectively. In general, we know that 4CDD/Fs and 8CDD/Fs are eluted in order first and last (or in the opposite order depending on the kind of packing material) from the column chromatographies such as silica gel, alumina, and activated carbon silica gel. Therefore, if the good recoveries of the surrogates of 4CDD/Fs and 8CDD/Fs are confirmed, it is predicted that 5-7CDD/Fs is also sufficiently collected. We examined the correlation between measured data for two kinds of surrogates and ten kinds of surrogates. For this requirement, we actually added the ten kinds of surrogates (it was one kind in each congener; $^{13}C_{12}$-OCDD was included for 8CDD/F) and $^{13}C_{12}$-1,2,3,4-TCDF to human milk (19 samples) at the same time and did a series of analyses. As a result, the values between each congener were in excellent agreement. Furthermore, an excellent correlation ($r=0.956$) was also obtained for TEQ among them (Figure 1). Based on these results, doing a common preprocessing operation in the GC/MS method and ELISA became possible by using $^{13}C_{12}$-1,2,3,4-TCDF, and $^{13}C_{12}$-OCDD as surrogates.

2. Simplifying of the preprocessing operation

The sulfuric acid washing requires the difficulty handling of concentrated sulfuric acid and also has the problem of waste fluid treatment. Therefore, the alkali decomposition method was employed as a preprocessing operation for ELISA. In addition, we examined the technique by

which the multi-layer silica gel column that occupied the important position as a cleanup operation was easily done. A conventional multi-layer silica gel column needed 5 kinds of packing materials, and the packing operation of the column was extremely complex and required skill to complete the work. We then examined omitting the various kinds of packing materials in the multi-layer column. It was thought that impurities were significantly excluded before the multi-layer silica gel column treatment because the fat extracted from the human milk was decomposed in alkali. Therefore, it has been understood that 1/5 or less of the previous amount of the packing materials is needed, and that neither the silica gel impregnated silver nitrate nor the silica gel impregnated potassium hydroxide are necessary. Moreover, 22% or 44% sulfuric acid silica gel was sufficient for the sulfuric acid silica gel. On the other hand, impurities such as pigment and relatively polar substances from the sample still exist in the hexane extract after the alkaline treatment. Therefore, carbonization occurs in the upper part of the column as the impurities react with sulfuric acid when this extract comes in contact with the sulfuric acid silica gel. We understood that most of the impurities with a comparatively large polarity of sample origin were adsorbed by the silica gel by accumulating a small amount of silica gel on the upper part of the sulfuric acid silica gel. This accumulation was able to considerably reduce the load of the sulfuric acid silica gel. On the other hand, the coexisting material with a large polarity, newly generated by the sulfuric acid silica gel processing, was occasionally eluted from the column and was mixed in the dioxin fraction. When another silica gel layer was accumulated on the lower part of the sulfuric acid silica gel, it was understood that the above-mentioned coexisting material was removed. On the basis of these results, it has been understood that the column made of three-layers with two kinds of packing materials is sufficient for cleanup.

In this research, the performance of the prototype, which was filled with the three-layer sulfuric acid silica gel in the above-mentioned disposable cartridge for reducing the packing operation labor, was also evaluated. As a result, the elution behavior of dioxins, the recoveries of the surrogates, and chromatograms in real samples were quite similar compared with the above-mentioned three-layer sulfuric acid silica gel column. In addition, it was confirmed that there was no problem during practical use. Accordingly, a prompt analysis became possible by using the handy cartridge column developed in this study.

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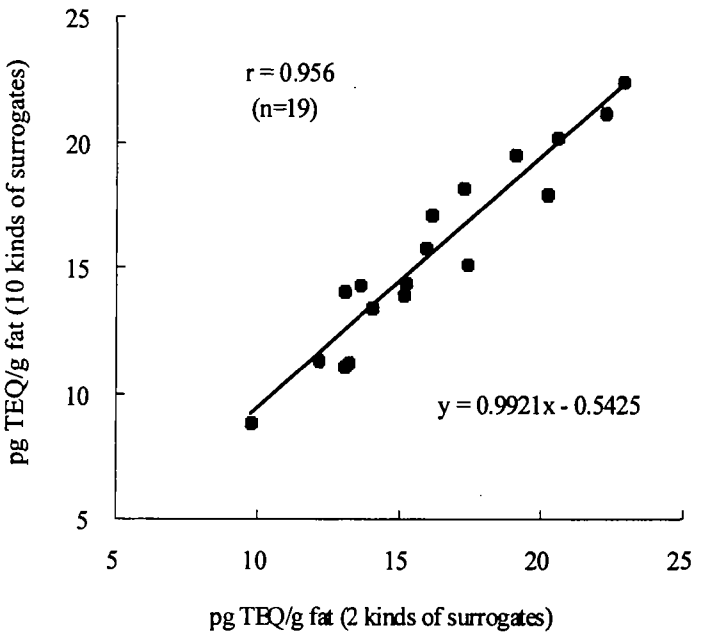

Figure 1. Correlation between data measured with 2 kinds of surrogates and 10 kinds of surrogates