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Enzyme-Linked Immunosorbent Assay Toxicity Evaluation Method for Dioxins in Human Milk

K. Saito,[1] M. Takekuma,[1] M. Ogawa,[1] S. Kobayashi,[1] Y. Sugawara,[2]
M. Ishizuka,[2] H. Nakazawa,[3] Y. Matsuki[4]

[1] Dioxin Research Group, Saitama Institute of Public Health, 639-1, Kamiokubo, Saitama 338-0824, Japan
[2] Cosmo Research Institute, 4-9-25, Shibaura, Minato-ku, Tokyo 108-8564, Japan
[3] Department of Analytical Chemistry, Hoshi University, 2-4-41, Ebara, Shinagawa-ku, Tokyo 142-8501, Japan
[4] Food and Drug Safety Center, Hatano Research Institute, 729-5, Ochiai, Hadano, Kanagawa 257-8523, Japan

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The contamination of food and the ecosystem by dioxins and its resultant effects on our health have been drawing much attention from the public. Thus, the investigation of human exposure to dioxins is an urgent and important task for the government. Since the Law Concerning Special Measures against Dioxins (Environmental Agency of Japan, 1999) became effective in Japan in 1999, the number of substances to be sampled and measured are expected to increase.
Conventionally, high resolution gas chromatography/mass spectrometry (HR-GC-MS) has been used for the measurement of dioxins. However, a GC-MS method has a drawback in that it requires a complicated cleanup procedure for measurement of all types of samples as well as it is very time-consuming and extremely expensive to perform. Thus, the development of a method for measuring dioxins, which is inexpensive, easy to perform, and highly sensitive, 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. However, most of the reports dealt with standard substances (Stanker et al. 1987; Sugawara et al. 1998; Carlson et al. 1998), fly ash (Harrison et al. 1997; Zennegg et al. 1998; Li et al. 1999), soil (Harrison et al. 1997; Harrison et al. 1998; Harrison et al. 1999), sediment (Li et al. 1999), and chimney soot (Zennegg et al. 1999), which contained dioxins in high concentrations. There has been no report on a practical assay that can deal with biological samples containing dioxins in extremely low concentrations such as human milk and blood. The conventional ELISA has been considered as a simple screening method, but less reliable compared with the GC-MS method.

In this report, we constructed a basic strategy for the development of a toxicity evaluation method for dioxins in human milk by ELISA. Also, we selected an optimal isomer to be detected, which is the key factor in the development of this ELISA method. Furthermore, to make the proposed ELISA method compatible with the conventional GC-MS method, it was tried to use the same preprocessing operation, and it was also examined if an extremely complicated conventional preprocessing operation could be simplified. For these objectives, we made a

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Correspondence to: K. Saito

prototype of a three-layer H_2SO_4/silica-gel cartridge that took the place of a multi-layer silica-gel column, and also evaluated its applicability for practical use.

METHODS AND MATERIALS

All of the dioxin standards were from Wellington Laboratories and were diluted with decane to the appropriate concentrations. Most of the organic solvents such as hexane, dichloromethane, toluene and diethylether were of dioxin analysis quality from Kanto Kagaku (Tokyo, Japan) or Wako Pure Chemicals (Osaka, Japan). Silica-gel impregnated activated carbon, and 44 % H_2SO_4/silica-gel were from Wako Pure Chemicals. The prototype of the three-layer H_2SO_4/silica-gel column packed in a disposable cartridge tube (inside diameter 15 mm x length 75 mm; made of polypropylene) was made by a special request to Supelco (USA). The cartridge was washed with 40 ml of hexane prior to use.

Three-layer H_2SO_4/silica-gel column: one g silica-gel, 2 g 44% H_2SO_4/silica-gel, 0.5 g silica-gel, and 1 g Na_2SO_4 were sequentially layered from the lower layer into a glass column having an inside diameter of 1cm by slurry packing with hexane. The column was then washed with 40 ml of hexane prior to use.

Human milk samples, at about 30 days after birth, were collected from 100 Japanese primiparae whose mean age was 28.5 years old.

As for the analysis of dioxin in human milk by the GC-MS method, ten kinds of stable isotopes of the 2,3,7,8-substituted congeners of PCDDs and PCDFs, and $^{13}C_{12}$-1,2,3,4-TCDF were added as surrogate after the fat was extracted from the human milk according to the Official Methods of Analysis of the AOAC International (Association of Official Analytical Chemists, 1995). The fat was then subjected to concentrated sulfuric acid washing and 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-MS using a JEOL JMS-700 MStation mass spectrometer coupled to a HP-6890 HR-GC with a capillary column of DB-17HT (30 m x 0.25mm i.d., film thickness 0.15 um; J & W Scientific) for the isomer specific separation. The GC program was as follows: 150°C for 1 min, increased 20°C /min to 220°C and subsequently 4°C /min to 280°C, then maintained for 11.5 min. Helium was used as the carrier gas. The injector temperature was 280°C and the GC-MS interface temperature was held at 280°C. The MS was operated in the selected ion monitoring mode with a mass resolution of 10000, and the electron impact ionization energy was 38 eV, with an ion source temperature of 260°C. Quantification was done by the isotopic dilution method, i.e. the PCDD/Fs congeners were quantified by comparison with their respective reference $^{13}C_{12}$-labeled standards in the following two ways; in one way, one kind of stable isotope in each congener of the PCDD/Fs was used as the internal standard, and in the other method, $^{13}C_{12}$-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, and then the PCDD/Fs concentrations were calculated on the fat basis. The toxicity equivalent quantity

(TEQ) was calculated using WHO-TEF(1998).

As for ELISA, the fat was extracted from human milk, and surrogates were added to the fat, and then 40 ml of 1N-KOH/ethanol was added and stirred. The solution was then allowed to stand at room temperature overnight. The alkaline solution was diluted with 40 ml of water, followed by liquid-liquid extraction with 40 ml of hexane (twice). After the hexane layer was dehydrated and concentrated, the extracted material was processed with the three-layer H_2SO_4/silica-gel column using 40 ml of hexane as the eluent. The eluate was split equally into two aliquots; one was for ELISA and the other was for the GC-MS analysis. The eluate for ELISA was dried by a nitrogen stream, and the residue was re-dissolved into 60 μl of MeOH-DMSO (1:1) with 100 ppm Triton X-100. The ELISA was done according to the method previously reported by Sugawara et al. (Sugawara et al. 1998). The antiserum used was the same one that Sugawara et al. (Sugawara et al. 1998) had already developed and reported.

RESULTS AND DISCUSSION

We consider that an ELISA suitable for the measurement of dioxins in biological samples should satisfy the following requirements:

1) The ELISA should be able to evaluate the toxicity instead of providing only a simple screening method,

2) The ELISA should share a common pre-treatment procedure with the conventional GC-MS method so that valuable biological samples are effectively used, and also be compatible with the GC-MS. Moreover, the ELISA should not require a complicated pre-treatment procedure.

3) The data obtained from actual samples (human milk) by the ELISA should be highly correlated with those obtained by the GC-MS method.

In this report, we will focus on 1) and 2), and 3) will be reported in another paper.

In this study, we constructed a basic strategy for the development of a highly sensitive and simple method for the measurement of dioxins in human milk by ELISA and for the evaluation of the method as a toxicity evaluation method. We presented ELISA as a method for directly evaluating toxicity and not as a simple screening method. This is a new approach to ELISA. That is, after the fat is extracted from human milk, a simple pre-treatment is performed on the fat, and then half of the pre-treated fat as a testing solution is immediately evaluated for its toxicity by ELISA. If the concentration of each isomer needs to be measured or the TEQ needs to be confirmed, the results obtained by ELISA are feed-back, and the remaining half of the sample is subjected to cleanup, followed by measurement using the GC-MS method. This method is considered to increase the additional value of the data obtained by ELISA and enable ELISA to be compatible with the GC-MS method.

We determined the isomer to be targeted for the construction of a toxicity evaluation method. Since ELISA has the property that it can perform a specific measurement only on a specific chemical substance, it cannot separate or

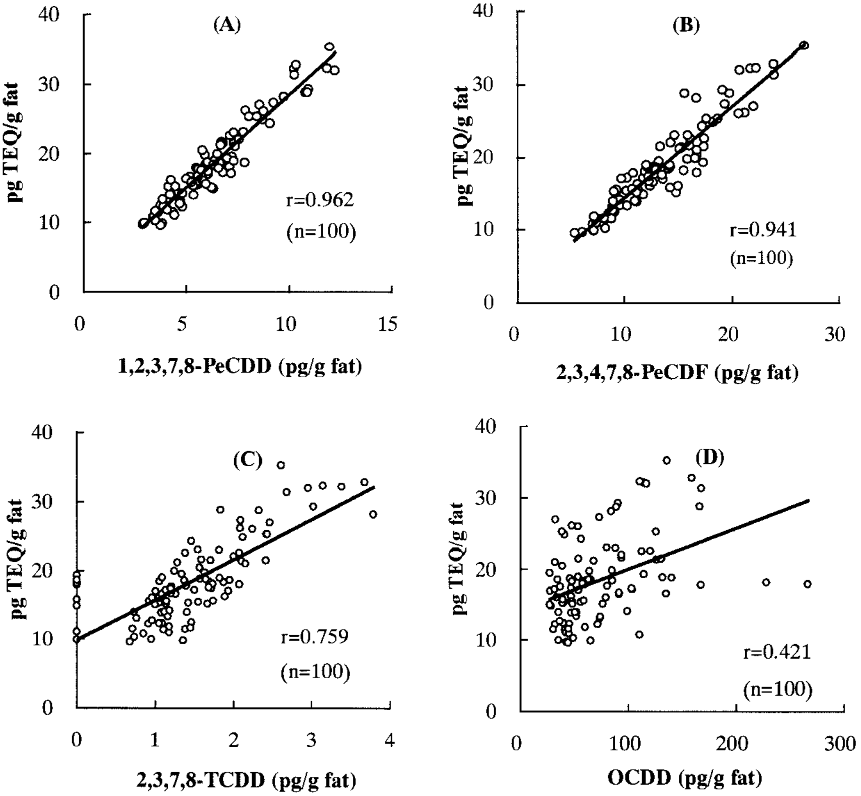

Figure 1. Correlation between TEQ of PCDD/Fs and each isomer ;
(A) 1,2,3,7,8-PeCDD, (B) 2,3,4,7,8-PeCDF, (C) 2,3,7,8-TCDD, and (D) OCDD.

determine different isomers unlike the GC-MS method. However, by using an antibody that is highly responsive to an isomer having a high toxicity and poorly responsive to an isomer having a low toxicity, the results by ELISA provide not only the detection values but also the degrees of toxicity. In order to develop a toxicity evaluation method by ELISA based on this concept, it was necessary to examine the relationship between the various isomers detected from biological samples and the TEQs. Accordingly, 100 samples of human milk were analyzed by the GC-MS method to find isomers that are highly correlated with the TEQ and detected in large amounts. As shown in Figure 1, either isomer of 1,2,3,7,8-PeCDD or 2,3,4,7,8-PeCDF was found to highly correlate with the TEQ (r=0.962, r=0.941, respectively). The most likely reason for this is that both have high toxicity equivalent factors (TEF) and are detected in relatively large amounts. In contrast, a typical dioxin isomer, 2,3,7,8-TCDD, was found not to correlate as highly with the TEQ (r=0.759) as the above two isomers. This is probably due to the smaller amount of detected 2,3,7,8-TCDD. OCDD had the highest concentration among the isomers detected from human milk, but showed a low correlation with the TEQ (r=0.421) because of its extremely low TEF of 0.0001.

Based on these results, the optimal isomers to be targeted for measurement by ELISA were determined to be 1,2,3,7,8-PeCDD and 2,3,4,7,8-PeCDF.
The ELISA used in this investigation showed that 1,2,3,7,8-PeCDD has almost the same cross-reactivity to 2,3,7,8-TCDD (Sugawara et al., 1998). However, the other isomers had a low reactivity in the ELISA. Therefore, it is considered that this ELISA detected the total amount of 2,3,7,8-TCDD and 1,2,3,7,8-PeCDD. These results show that the ELISA worked advantageously for the toxicity evaluation based on the TEQ, since the TEF of both 2,3,7,8-TCDD and 1,2,3,7,8-PeCDD is 1 according to the latest WHO-TEF (1998).

The conventional GC-MS method for dioxin analysis needs appropriate surrogates spiked prior to sample cleanup. However, as the surrogates usually used are 2,3,7,8-substituted $^{13}C_{12}$-congeners, which react with an antibody used for ELISA, the test solution prepared for the GC-MS method cannot be applied to ELISA. Thus, in this study, we decided to examine a surrogate that does not cause a cross reaction with ELISA, and that also enables a GC-MS method to measure the isomers with good accuracy. In order to achieve this objective, the following requirements should be satisfied.

1) The cross reactivity in ELISA should be extremely small.

2) As the conventional GC-MS method separately measures two groups of congeners; one consists of 4-6CDD/Fs and the other consists of 7-8CDD/Fs, the surrogate should be made to enter by one kind in each group.

3) Quantification values measured by the selected surrogate should agree with those measured by a conventional method.

The antibody used in this study showed a tendency to strongly react with congeners of high TEFs (Sugawara et al., 1998). 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 these requirements. The TEFs of these two congeners are 0 and 0.0001, respectively. The two congeners also showed less cross-reactivity in the ELISA we used. In general, we know that 4CDD/Fs and 8CDD/F are eluted from the column chromatographies, such as silica-gel, alumina, and activated carbon silica-gel, in the order first and last, respectively, or possibly in the opposite order if the chromatography packing material is different, when the chromatography is carried out as a conventional sample cleanup operation. Therefore, if good recoveries of the surrogates of 4CDD/Fs and 8CDD/F are confirmed, it is expected that the 5-7CDD/Fs are also collected in sufficient amounts.
As for requirement No.3, we made a comparative study between the use of ten kinds of surrogates and the two surrogates selected above. For that, we added the ten kinds of surrogates (one kind for each congener) and the two surrogates selected above to 19 samples of human milk at the same time and did a series of analyses. As shown in Table 1, the resulting correspondence between the values for each congener was excellent; furthermore, excellent correlation (r=0.956) was also obtained between both for the TEQ (Figure2). Based on these results, using $^{13}C_{12}$-1, 2, 3, 4- TCDF and $^{13}C_{12}$-OCDD as surrogates makes a common

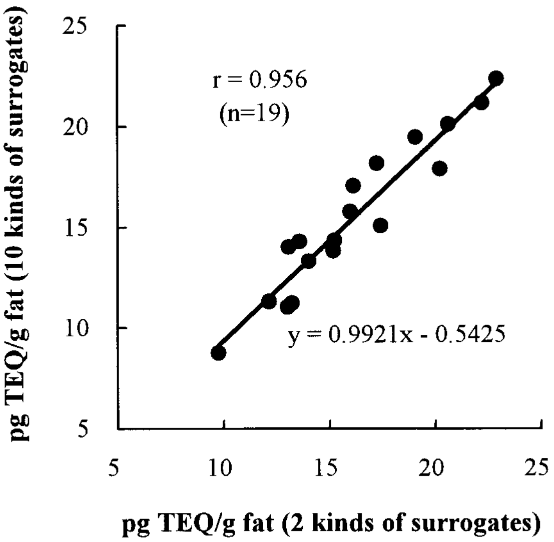

Figure 2. Correlation between TEQ data calculated with 2 kinds of surrogates and 10 kinds of surrogates.

Table 1. Correlation between PCDD/Fs data calculated by 2 kinds of surrogates and by 10 kinds of surrogates

	2 kinds	10 kinds	Correlation Coefficient
Dioxin isomers	Mean (pg/g fat)		(n=19)
2,3,7,8-TCDD	1.6	1.5	0.982
1,2,3,7,8-PeCDD	10.0	7.3	0.958
1,2,3,4,7,8-HxCDD	2.4	2.3	0.977
1,2,3,6,7,8-HxCDD	19.2	18.5	0.872
1,2,3,7,8,9-HxCDD	3.9	3.9	0.985
1,2,3,4,6,7,8-HpCDD	11.3	9.4	0.936
OCDD	73.2	75.0	1.000
2,3,7,8-TCDF	0.4	0.7	0.991
1,2,3,7,8-PeCDF	0.6	0.8	0.996
2,3,4,7,8-PeCDF	10.4	13.6	0.955
1,2,3,4,7,8-HxCDF	4.5	4.6	0.978
1,2,3,6,7,8-HxCDF	5.3	5.5	0.960
2,3,4,6,7,8-HxCDF	3.4	3.6	0.969
1,2,3,7,8,9-HxCDF	0.3	0.2	0.997
1,2,3,4,6,7,8-HpCDF	3.3	3.0	0.932
1,2,3,4,7,8,9-HpCDF	ND	ND	—
OCDF	0.1	0.1	1.000

preprocessing operation for the GC-MS and ELISA methods possible.

Simplifying the preprocessing operation was studied, because conventional preprocessing operations such as sulfuric acid washing and various chromatographies for dioxin analysis of human milk require a complicated cleanup which are very time consuming. Particularly, the sulfuric acid washing has a difficulty in handling concentrated sulfuric acid and also has a problem in treating the waste fluid. Therefore, the alkali decomposition and the multi-layer silica-gel column processing method were employed as a preprocessing operation for ELISA. In this study, we examined a technique by which a multi-layer silica-gel column, which is important in a cleanup operation, can be simplified.

A conventional multi-layer silica-gel column is prepared by accumulating 7 layers with 5 kinds of packing materials, and also requires relatively large amounts of materials. In addition, the packing operation of the column is extremely complex and requires skill to accomplish. We then examined a way of omitting some of the packing materials used in the multi-layer column and reducing the amounts of the packing materials. It was considered that impurities were significantly excluded before the multi-layer silica-gel column treatment because the fat extracted from human milk was processed by decomposition in alkali. Therefore, it has been understood that a total amount of 1/5 or less of the packing materials is sufficient. In addition, we sequentially removed several of the accumulated packing materials, and examined the influence on the GC-MS chromatograms and on the recoveries of the surrogates. As a result, it has been shown that neither the silica-gel impregnated silver nitrate nor the silica-gel impregnated potassium hydroxide was necessary. Moreover, either 22% or 44% H_2SO_4/silica-gel was sufficient. On the other hand, impurities such as pigment and relatively polar substances from the sample still existed in the hexane extract after the alkaline treatment. Therefore, carbonization occurred in the upper part of the column as the impurities reacted with the sulfuric acid when this extract came in contact with the H_2SO_4/silica-gel. The impurities with a relatively large polarity of these sample origins were then adsorbed by the silica-gel by accumulating a small amount of silica-gel in the upper part of the H_2SO_4/silica-gel. This accumulation was able to considerably reduce the load of the H_2SO_4/silica-gel. On the other hand, the coexisting material newly generated by the H_2SO_4/silica-gel processing was occasionally eluted from the column and was mixed in the dioxin fraction. When another silica-gel layer was accumulated in the lower part of the H_2SO_4/silica-gel, the above-mentioned coexisting material was removed. On the basis of these results, it has been shown that the column having three layers with two kinds of packing materials is sufficient for cleanup. To remove moisture in the sample, a small amount of sodium sulfate is usually used, but it is not always necessary because moisture in the sample is dehydrated before the column chromatography.

In this study, the performance of the prototype, which was filled with the three-layer H_2SO_4/silica-gel in the above-mentioned disposable cartridge for saving labor during the packing operation, was also evaluated. As a result, the elution behavior of dioxins, the recoveries of the surrogates, and the chromatograms of real samples were similar to those when the above-mentioned three-layer H_2SO_4/silica-gel column was used. In addition, it was confirmed that there was no problem during practical use. Accordingly, it has been shown that using the simplified cartridge column developed in this research makes a prompt analysis possible.

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