DEVELOPMENT OF DIOXIN TOXICITY EVALUATION METHOD IN HUMAN MILK BY ENZYME-LINKED IMMUNOSORBENT ASSAY (PART IV: A STUDY ON SIMPLIFICATION OF PRETREATMENT)

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Introduction

Due to the social concern over the contamination of the ecosystem and the food system by dioxin as well as its effect on our health, investigation of the current state of human exposure to dioxin and the resultant contamination has been demanded. For dioxin analysis, establishing an appropriate assay and a system for controlling accuracy are indispensable to secure the reliability of the analysis. In addition, there has been an increasing demand for a simple assay for quick screening. We investigated an enzyme-linked immunosorbent assay (ELISA) that can serve as a practical analysis of dioxin in human milk[1-3], and showed that ELISA serves not only as a simple screening method but also as a direct toxicity evaluation method. We also made the pretreatment of the ELISA the same as that of the conventional GC/MS method in order to perform both methods efficiently. In this study, to further simplify the pretreatment in the ELISA, we investigated a method in which a disposable cartridge is used to extract lipid from human biological samples (vernix caseosa). We also studied the practicality of using a prepacked multi-layer silica gel cartridge to clean up lipid extracted from human milk and vernix caseosa in a single step.

Methods and Materials

Simple lipid extraction from Biological samples

A lipid sample (corn oil, lard, butter) of 2-3 g was used. Twenty times the weight of the sample of sodium sulfate was added to the sample and mixed well to dehydrate and disperse it. The sample was then charged into a PP cartridge having a content volume of approximately 60 mL (internal diameter 26 mm x 120 mm ; product of Supelco). By use of an attached frit, the charged content was covered and fixed, and then eluted with ether:petroleum ether (1:1) mixture (35 mL). The elution rate was adjusted to be 2-3 mL/min with a PP mini stopcock attached to the tip of the cartridge. The eluate was subjected to a rotary evaporator or nitrogen purge to remove the solvent, and the lipid was obtained.

Simple cleanup for ELISA using a multi-layer silica gel cartridge

The extracted lipid was dissolved in a small amount of hexane, and then charged into a prepacked multi-layer silica gel cartridge (product of GL Science; Fig. 1) well washed with hexane in advance. After 120 mL of hexane was added for elution, the solvent in the eluate was removed, and the resultant eluate was washed into a conical test tube with a small amount of hexane, to which 60 μL of 0.1% Triton X- 100 (MeOH solution) was added, followed by nitrogen purge. Measurement by ELISA was performed as described in Sugawara et al.[3]

GC/MS measurement

The following two methods of GC/MS analysis of dioxin in human milk were compared. In both methods, lipid was extracted according to the conventional method. Then in the first method, the extracted lipid was subjected to alkali decomposition, a three-layer sulfuric acid silica gel column treatment, and then activated charcoal column treatment, while in the second method, the extracted lipid was directly subjected to a multi-layer silica gel cartridge followed by activated charcoal column treatment.

DB-17HT was used as a capillary column for separation in GC, and JEOL JMS-700 was used as a high resolution MS. The resolution was set at 10000, and SIM measurement was performed according to lock-mass method. Dioxin isomer concentrations were calculated on fat basis, and TEQ was calculated using WHO-TEF(1998).

Results and Discussion

Simple lipid extraction from biological samples

The toxicity equivalent quantity (TEQ) per gram of lipid is used for the evaluation of dioxin in biological samples such as human milk and blood. Thus, it is essential to extract lipid from samples prior to the dioxin analysis. However, the conventional method for extracting lipid involves complicated procedures and requires a substantial amount of equipment. In order to remove this drawback, we aimed to develop a simple method for extracting lipid from biological samples. In this study, we studied a simple method for extracting lipid from vernix caseosa and adipose tissues. Vernix caseosa is a secrete from a fetus, mainly composed of lipid, and can be obtained from the body surface of a fetus only at the time of birth. By analysing vernix caseosa, the degree of contamination of a fetus by dioxin can be estimated. However, since vernix caseosa is very difficult to obtain, corn oil (liquid), lard (semi-solid), and butter (solid) were selected as simulated samples in this study. Sodium sulfate was added to remove moisture in the samples and to increase the contact area with an organic solvent by dispersing the sample. The required amount of sodium sulfate was found to be about 20 times the weight of each sample. Ether: petroleum ether (1:1) mixture, which has previously been used for extraction of lipid from human milk, was used as an organic solvent for eluting the lipid. A fraction of the eluate of each sample was collected every 5 mL to study the elution pattern and the lipid in each sample was found to be eluted with an addition of the organic solvent in an amount of no more than 30-35mL. The overall recovery of corn oil and lard was almost 100%, and that of butter was about 75%. However, the butter used in this study contained ingredients such as non-fat milk solids in addition to vegetable fat and animal fat, which amounted to 75% in total. Therefore, the effective overall recovery of butter is considered to be almost 100%. According to this extraction method, a sample dispersed in sodium sulfate, which is used as the solid phase, and an organic solvent are subjected to

chromatographic liquid-liquid distribution extraction. Thus, the extraction method can be performed more easily and quickly and shows a higher extraction efficiency than liquid-liquid extraction performed in the conventional batch operation. Accordingly, the extraction method is considered to be applicable to biological samples such as adipose tissues in addition to vernix caseosa.

Simple cleanup using a multi-layer silica gel cartridge

A cleanup method for analysis of human milk, in which alkali decomposition and a three-layer sulfuric acid silica gel cartridge are used, was reported at the dioxin symposium last year[2]. Although an excellent cleanup effect can be obtained by employing this method, it requires overnight alkali decomposition treatment (about 12 hours) and liquid-liquid distribution extraction using hexane. In this study, we aimed to develop a simpler cleanup method that allows extracted lipid to be treated directly using a multi-layer silica gel column without subjecting it to alkali decomposition. There used to be a drawback to this in that preparation of a multi-layer silica gel column required complicated procedures, but recently a prepacked multi-layer silica gel in a disposable cartridge has become available. Lipid extracted from human milk was dissolved in a small amount of hexane and then charged into the cartridge directly. When the weight of the lipid does not exceed about 2 g, it was found that impurities such as fat could be decomposed and removed by the fillers in the cartridge. Following the multi-layer silica gel cartridge treatment, the lipid was subjected to activated charcoal column treatment. The recovery of surrogate was obtained by GC/MS and found to be comparable to the excellent recovery obtained with the previous cleanup method, i.e. alkali decomposition, three-layer sulfuric acid silica gel column treatment, and activated charcoal column treatment (Table 1).

Next, we studied if the ELISA can measure a test solution treated with the multi-layer silica gel cartridge. The result showed that the value (the total value of 2,3,7,8-TCDD and 1,2,3,7,8-PeCDD) obtained by the ELISA was significantly lower than that obtained by GC/MS. A conceivable reason for this is that a false negative was shown in the ELISA, which is a competitive assay, because dioxin was incorporated into a trace amount of lipid present in the test solution. In our study conducted last year, we reported that 6 μg of Triton X-100 can make a trace amount of lipid present in samples dispersible and soluble. To the eluate from a multi-layer silica gel cartridge, Triton X-100 was added in an amount of 60 μg and 600 μg. The result showed that when Triton X-100 was added in an amount of 60 μg, the obtained value was greatly improved so as to show sufficient correlation with the value obtained by GC/MS. In contrast, when Triton X-100 was added in an amount of 600 μg, cloudiness and precipitation were observed when nitrogen purge concentration was performed, and a satisfactory result was not obtained by the ELISA. Thus, the optimal amount of Triton X-100 to be added is considered to be 60 μg.

Acknowledgement

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Reference

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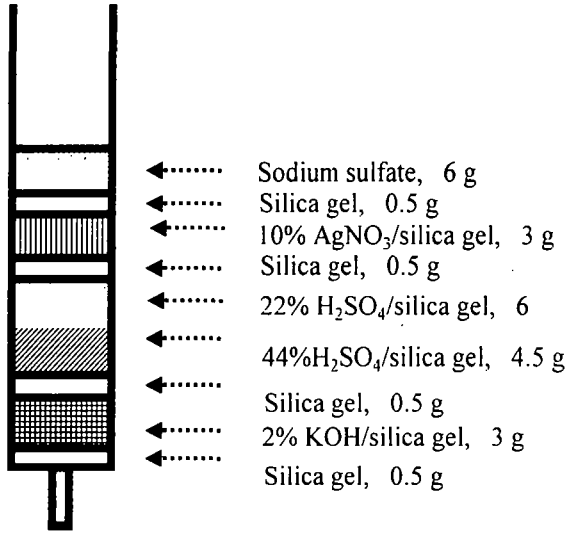

Figure 1. Multi-layer silica gel cartridge

Table 1. Comparison of the recovery between multi-layer silica gel cartridge method and alkari decomposition method

	Mean recovery (%)	
Dioxin congeners ($^{13}C_{12}$ isotope)	Multi-layer silica gel (n=5)	Alkali decomposition (n=5)
2,3,7,8-TCDD	81.3	81.2
1,2,3,4,6,7,8-HpCDD	77.4	69.7
2,3,7,8-TCDF	89.5	84.3
1,2,3,4,6,7,8-HpCDF	62.2	69.5

Dose concentration to human milk sample : 2 ppb