Determination of Organotins in Human Breast Milk by Gas Chromatography with Flame Photometric Detection

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An analytical method for the quantitative determination of monobutyltin (MBT), dibutyltin (DBT), tributyltin (TBT) and triphenyltin (TPhT) compounds in human breast milk is described. After the addition of surrogates (deuterium derivatives), milk samples were extracted with hexane-diethyl ether (4 : 6) in the presence of HCl and NaCl. Each extract was purified by cation exchange chromatography and treated with Grignard reagent to yield ethyl derivatives, which were determined by gas chromatography (GC) with flame photometric detection operated in the tin mode (610 nm). These organotin chlorides, spiked to milk at 12.5, 25, and 50 ng/ml (ppb), were recovered within a range of 85 to 105%. Detection limits were 1.3 ng/ml for DBT, TBT, and TPhT, and 2.5 ng/ml for MBT. This analytical method was used to determine organotins in about 70 breast milk samples obtained from mothers who had given birth within the previous week. DBT dichloride levels varied from undetectable to 9.5 ng/ml in human milk from mothers who habitually ate fish, however, the other organotins were not detectable. No significant difference was observed in DBT contents between mothers who ate fish more than twice a week and those who ate fish less than once a week. Thus, since the levels of organotin even in the milk of mothers who liked to eat fish were very low, human breast milk should be considered safe for feeding infants, at least concerning with regard to the possible transmis-

Key words —— human breast milk, tributyltin, dibutyltin, monobutyltin, organotins, gas chromatography/flame photometric detector

INTRODUCTION

Organotin compounds have been extensively used as biocides, especially in antifouling paints. 1) The most widely used compounds, tributyltin (TBT) and triphenyltin (TPhT), cause deleterious biological effects, such as shell abnormalities in oysters and imposex in gastropods, even at concentrations below a few ng/l (ppt). 2) In many countries, the use of TBT and TPhT compounds as antifouling paints for small boats is now restricted by law. However, it has been reported that about 69% of all large ships still use these compounds. 3) In addition, large amounts of other organotin compounds, such as dibutyltin (DBT) and dioctyltin, are used as stabilizers for plastics such as polyvinyl chloride (PVC). Environmental contamination by these compounds may cause serious problems. In fact, recent studies have concluded that organotin levels in water and sediment samples from marine environments remain relatively high, and continue to pose a toxicological risk to marine organisms, 4–8)

Through the consumption of polluted seafood, it is possible that humans can ingest unhealthy amounts of organotin. To evaluate the toxicity in newborn children, it is particularly important to monitor the trace amounts of organotins that might contaminate human breast milk. In mammals, organotins are suspected to be transferred from the mother to the offspring via the placenta and breast milk, by analogy with other hydrophobic pollutants such as dioxins, polychlorinated biphenyls (PCBs), and p,p′-chlorodiphenyltrichloroethane (DDT). Therefore, organotin levels in mothers are of great concern with regard to the healthy growth of fetuses and infants. However, there has been no previous report on the analysis of organotin compounds in human breast milk.

In this report, we describe a relatively simple and reliable analytical method for determining levels of organotins in human breast milk by gas chromatography (GC) with flame photometric detector (FPD) combined with a pretreatment procedure, i.e., a solvent extraction, cleanup by cation ion exchange chromatography, and treatment with Grignard reagent. These organotin chlorides, spiked to milk at 12.5, 25, and 50 ng/ml (ppb), were recovered within a range of 85 to 105%. Detection limits were 1.3 ng/ml for DBT, TBT, and TPhT, and 2.5 ng/ml for MBT. This analytical method was used to determine organotins in about 70 breast milk samples obtained from mothers who had given birth within the previous week. DBT levels varied from undetectable to 9.5 ng/ml in human milk from mothers who habitually ate fish, however, the other organotins were not detectable. No significant difference was observed in DBT contents between mothers who ate fish more than twice a week and those who ate fish less than once a week. Thus, since the levels of organotin even in the milk of mothers who liked to eat fish were very low, human breast milk should be considered safe for feeding infants, at least concerning with regard to the possible transmission of organotin compounds.
exchange chromatography, and ethylation by Grignard reagent. This method was applied to determine organotin levels in dozens of human breast milk samples. The analytical results suggest that human breast milk is largely uncontaminated by organotin compounds.

MATERIALS AND METHODS

Materials —— Human breast milk samples were obtained as gifts from 67 volunteers who had given birth over the previous week in Okayama and Kagawa Prefectures in 2002. The samples from Okayama were divided into 2 groups; one group \((n = 31)\) of mothers ate fish more than twice a week and the other group \((n = 5)\) ate fish less than once a week. All other reagents were of the highest quality available.

Standards —— Monobutyltin (MBT) trichloride was purchased from Aldrich Chemical Co. (Milw., Wi, U.S.A.). MBT trichloride-d_9 (surrogate), DBT dichloride, TBT chloride, TBT chloride-d_27 (surrogate), and TPhT chloride were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). DBT dichloride-d_{18} (surrogate) and TPhT chloride-d_{15} (surrogate) were purchased from Hayashi Pure Chemical Industries, Ltd. (Osaka, Japan).

GC —— Shimadzu Model GC-2010 (Shimadzu Co., Kyoto, Japan) equipped with a FPD (FPD-2010) operated in tin (Sn, 610 nm) mode; Chrompack CP-Sil 8CB Low Bleed/MS capillary column, 0.25 mm id × 30 m, coated with 0.25 μm film (Chrompack, Middelburg, The Netherlands). Operating conditions: injection port, 280°C, detector. 300°C, column oven—initial 50°C, held 2 min, heated at 7°C/min to 120°C, heated at 10°C/min to 200°C, heated at 5°C/min to 250°C, heated at 20°C/min to 300°C, hold 2 min; helium carrier gas, 2.24 ml/min; hydrogen, 140 ml/min; air, 100 ml/min; sample injection volume, 1 μl; splitless injection mode; splitter opened after 30 sec.

Extraction and Cleanup —— Samples were extracted by the Method of Analysis in Health Science, with slight modification. To test recovery, 50 μl hexane containing 50–200 ng of MBT trichloride, DBT dichloride, TBT chloride, and TPhT chloride was added to 4 ml of breast milk just before extraction.

The EtOH solution was subjected to CEXC (with Amberlite CG-120 Type 1, in a 10 mm id. × 10 cm column compressed to ca. 2 cm bed length) and washed with 5 ml of EtOH, and the adsorbed fractions were then eluted with 15 ml of 12 M HCl—MeOH (1 : 9). The eluted samples were evaporated to near 2 ml under 25°C on a rotary evaporator, and the concentrated eluate was extracted with 15 ml of water, 2 g of NaCl, and 15 ml of hexane—diethyl ether (4 : 6). The aqueous solution was extracted again with 15 ml of hexane—diethyl ether (4 : 6). The combined organic layers were dried over anhydrous Na_2SO_4, and the residual solvents were removed on a rotary evaporator under 25°C to near dryness. The residue was dissolved in 3 ml of hexane—diethyl ether (4 : 6), and concentrated to ca. 1 ml under a stream of nitrogen gas. One ml of Grignard reagent was added and the mixture was allowed to stand for ca. 30 min. Next, 8 ml of 0.5M-H_2SO_4 was added under cooling in ice, followed by the addition of 8 ml of water and 5 ml of hexane, and the mixture was shaken vigorously. The organic layer was separated and the aqueous layer was extracted again with 5 ml of hexane. To the combined hexane extracts was added ca. 1 g of Na_2SO_4, and the mixture was allowed to stand for 30 min before being filtered through cotton wool. The filtrate was evaporated at 25°C to near dryness, and the residue was dissolved in 1 ml hexane. One μl aliquots were used for GC-FPD analysis.

Fifty ng of each surrogate was added to 4.0 ml of breast milk in a funnel flask, and the milk was then extracted with 25 ml of 3 M HCl (NaCl saturated), 1.5 g of NaCl, and 25 ml of hexane—diethyl ether (4 : 6) for 30 min. The mixture was centrifuged at 10000 rpm (20000 × g) for 10 min, and the organic layer was pooled. The residues and the aqueous layer were extracted again with 25 ml of hexane—diethyl ether (4 : 6) for 30 min. After the mixture was centrifuged again under the same conditions, the organic layer was pooled. The combined organic layers were washed with 12 ml of NaCl-saturated solution. The organic layer was then dried over anhydrous Na_2SO_4, and the residual solvent was removed on a rotary evaporator under 25°C to near dryness (MBT trichloride, DBT dichloride, TBT chloride, and their ethyl derivatives are volatile; therefore, it is necessary to concentrate the sample solution to near dryness at low temperature throughout the procedures). Finally, the sample residue was dissolved in 10 ml of EtOH to give a solution ready for cation exchange column chromatography (CEXC) cleanup.
RESULTS AND DISCUSSION

Detection and Ethylation

Electron capture detector (ECD) and FPD have been used in the GC determination of trace levels of alkyltin compounds. ECD detects alkyltin hydrides and chlorides with high sensitivity; however, these compounds tend to be adsorbed on the column packing and severe sample cleanup is required for analysis by ECD/GC without detector deterioration.\textsuperscript{10–12} We used FPD, which is sensitive to tetra-alkyltins and needs only a simple cleanup compared with ECD. Figure 1 shows gas chromatograms of MBTEt, DBTEt, TBTEt, MBTEt-\textsubscript{d9}, DBTEt-\textsubscript{d18}, and TBTEt-\textsubscript{d27} (ethyl derivatives) for FPD operated in the tin mode. These tetra-alkyltins were prepared by the reaction of MBT trichloride, DBT dichloride, TBT chloride, MBT trichloride-\textsubscript{d9}, DBT-\textsubscript{d18}, or TBT chloride-\textsubscript{d27} with Grignard reagent (ethylmagnesium bromide). These ethyl derivatives showed a high response and good shapes, and good separation between organotins and the corresponding surrogates. Therefore, ethyl derivatization was used in our method.

Loss of Organotin During the Concentration Procedure

Organotin chlorides and their alkyl derivatives are fairly volatile and are lost during routine concentration procedures.\textsuperscript{13} Figure 2 shows that the loss of MBT trichloride, DBT dichloride, and their ethyl derivatives greatly increased as time after dryness increased. To prevent the loss of these compounds, concentration to near dryness at a low temperature was applied in the present proposed method.

Extraction and Clean Up

Extraction was performed according to the procedure described in “Qualitative and quantitative analysis of DBT and TBT by GC” in the Method of Analysis in Health Science\textsuperscript{9} with some modification as follows.

First, the volume of solvents used for extraction was decreased to about 25%, and the organic phase was then washed with 12 ml of NaCl-saturated solution instead of 50 ml of water to prevent the loss of organotin chlorides, especially MBT chloride. Moreover, in contrast to the use of anion and cation exchange chromatography in conventional procedures\textsuperscript{9} for fish samples, a clean-up step using anion exchange chromatography (MCI GEL CAPO 8P) was unnecessary for these milk samples which contained fewer impediments to analysis than fish samples.

Sensitivity and Detection Limit

The FPD (Shimadzu FPD-2010) used here is a high-sensitive type with a reflecting mirror and a lens. The detection limits for DBT, TBT, and TPhT compounds were 5 pg, corresponding to about 1.3 ng/ml (ppb) of each organotin chloride on a milk basis, which is comparable to the detection limits for the electron capture detector.\textsuperscript{10,11} For MBT compounds, the detection limit was 10 pg, corresponding to 2.5 ng/ml MBT trichloride on a milk basis. The addition of about 50 mg Na\textsubscript{2}SO\textsubscript{3} to 1 ml of the hexane solution prevented the loss of ethyl
derivatives during storage. Since halogen decomposes tetra-alkyltin, Br₂ derived from the reaction of Grignard reagent with organotin chlorides may affect on the stability of ethyl derivatives.\(^{14}\)

Table 1 shows the recoveries of MBT trichloride, DBT dichloride, TBT chloride, and TPhT chloride spiked to milk at 12.5, 25, and 50 ng/ml. These organotin chlorides were recovered within ranges of 85 to 105%.

**Procedures**

Based on the above results, a recommended procedure (described in Materials and Methods) was established for the determination of organotin compounds in human breast milk by GC/FPD.

**Analytical Results**

Figure 3 shows gas chromatograms for typical breast milk (A) and a DBT-contaminated sample (B), together with a chromatogram (C) for the same contaminated sample, in which the final solution to be injected was concentrated to 100 µl, instead of 1 ml, under nitrogen. A small peak of DBT appeared just after the peak position of DBT-\(d_{18}\) as a surrogate in the chromatogram (B). The ca. 10 fold-concentrated solution gave a definite peak of DBT (C), indicating that such concentration greatly improves the sensitivity and detection limit in organotin analysis.

Table 2 summarizes the analytical results for organotin compounds in 67 samples of breast milk. Of the 67 samples, only 11 showed detectable levels (2.5–9.5 ng/ml) of DBT compounds, and in the vast majority of samples these compounds were undetectable (less than 1.3 for DBT, TBT, and TPhT compounds, and less than 2.5 ng/ml for MBT compounds, respectively). Among the samples from Okayama Prefecture, no significant difference was observed in the DTB-detectable ratio (number of detectable samples per number of samples) between mothers who ate fish more than twice a week (five detectable/31 = 0.16) and those who ate fish less than once a week (one detectable/5 = 0.20). This result clearly suggests that even for mothers who consumed organotin-polluted fish, breast milk was not contaminated with organotin compounds. In contrast to dioxins and PCBs, organotin compounds in

![Fig. 3. Gas Chromatograms for Some Breast Milk Samples](image)

(A): a typical breast milk, (B): DBT-contaminated milk, (C): the same DBT-contaminated milk as in B, except that the final solution was concentrated to one-tenth of its original volume.

**Table 1.** Recovery of Organotin Compounds from Spiked Breast Milk

<table>
<thead>
<tr>
<th>Spiking level (ng/ml)(^{a})</th>
<th>MBT (×)</th>
<th>DBT (×)</th>
<th>TBT (×)</th>
<th>TPhT (×)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.5</td>
<td>85 ± 4</td>
<td>100 ± 6</td>
<td>103 ± 6</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>25</td>
<td>100 ± 6</td>
<td>92 ± 5</td>
<td>100 ± 3</td>
<td>99 ± 8</td>
</tr>
<tr>
<td>50</td>
<td>104 ± 3</td>
<td>88 ± 2</td>
<td>104 ± 2</td>
<td>92 ± 3</td>
</tr>
</tbody>
</table>

\(^{a}\) Amounts are expressed as concentration (ng/ml) of the breast milk (4 ml). \(^{b}\) Average of triplicate analyses.

**Table 2.** Dibutyl Tin Contents in Breast Milk Samples

<table>
<thead>
<tr>
<th>Breast milk samples</th>
<th>Mother’s home Prefecture</th>
<th>Frequency of fish meals a week</th>
<th>No. of samples</th>
<th>DBT content (ng/ml)</th>
<th>Analytical values</th>
<th>Average(^{c})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>Okayama</td>
<td>≥2 times</td>
<td>31</td>
<td>ND ((n = 26)), 2.5, 3.2, 3.3, 3.7, 5.0</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>Group B</td>
<td>Okayama</td>
<td>≤1 time</td>
<td>5</td>
<td>ND ((n = 4)), 4.5</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>Group C</td>
<td>Kagawa</td>
<td>unknown</td>
<td>31</td>
<td>ND ((n = 26)), 2.5, 3.2, 4.0, 5.5, 9.5</td>
<td>1.9</td>
<td></td>
</tr>
</tbody>
</table>

\(^{c}\) Values were calculated assuming that ND is 1.3 ng/ml. ND: 1.3 ng/ml (breast milk) for DBT.
the mother’s body were unlikely to be concentrated over a prolonged period and then excreted into her milk. Therefore, it is unlikely that infants will consume organotin compounds at concentrations higher than the toxic threshold via their mother’s breast milk, although there is still a problem regarding the sensitivity of infants to organotins. Further studies on the behavior of TBT and other organotin in the human body are needed.

This is the first report to show that human breast milk is largely uncontaminated by organotin compounds. The methods described here are efficient in that cleanup is simple, conventional FPD is used, and the recovery is sufficient as determined by the use of deuterium derivatives as surrogates. Furthermore, they are suitable for the analysis of organotin compounds in breast milk.

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REFERENCES