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Simple Method for Methylmercury Estimation in Biological Samples Using Atomic Absorption Spectroscopy

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Total mercury level in biological samples have often been analyzed using atomic absorption spectroscopy (AAS), following the conversion of all the mercury to atomic mercury vapor. On the other hand, analysis of methylmercury (MeHg) using an electron capture detector-gas chromatography (ECD-GC) has well been established. For ECD-GC analysis, the MeHg in samples must be extracted in toluene as its complex with chloride or dithizone. Here, we attempted to analyze MeHg content in rat tissues by the oxygen combustion-gold amalgamation method using AAS, following toluene extraction and back extraction to an aqueous medium. Since all the processes were carried out in a microtube using a micro homogenizing system, microtube mixer and a micro centrifuge, the time required to prepare 12 samples was as short as 30 min. Recoveries of MeHg added to rat brain, kidney and liver homogenates were 83.6–86.7%. Accordingly, a recovery factor of 0.85 was necessary to calculate MeHg content from the analytically obtained value. Using the present method and the previously established method for inorganic mercury quantification, the sum of methyl and inorganic mercury contents in MeHg-treated and non-treated control rat tissues fitted well the total mercury contents. The present method would be useful to estimate, at least roughly, MeHg content in biological samples using the same instrument as total mercury analysis.

Key words —— methylmercury quantification, atomic absorption spectroscopy, rat tissue, micro homogenizing system

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INTRODUCTION

Mercury is a global pollutant and is distributed in the natural environment including bioorganisms. Among naturally occurring mercury, methylmercury (MeHg) is the most hazardous chemical for human health, especially for the developing fetus. Exposure to MeHg mostly occurs via consumption of seafood, since it is accumulated in marine animals via a food chain. The safe exposure level of MeHg for pregnant women has recently been recommended by the 61st FAO/WHO Joint Experts Committee on Food Additives.[1] Accordingly, the MeHg contents have often been analyzed, as well as total (methyl plus inorganic) mercury contents in various samples. Analytical procedures for each of these mercurial species are quite different. Analysis of MeHg using electron capture detector-gas chromatography (ECD-GC) has been well established.[2,3] For ECD-GC analysis, the MeHg in samples must be extracted in toluene as its complex with chloride or dithizone. On the other hand, total mercury frequently has been analyzed using atomic absorption spectroscopy (AAS), following conversion of all the mercurial species to atomic mercury vapor. Conversion of mercury to its vapor is performed by reductive reaction with $SnCl_2$ or by combustion above 800°C.[4–6] Accordingly, two instruments, ECD-GC and AAS, are necessary to quantify both MeHg and total mercury, since organic solvents such as toluene must be avoided for AAS. Previously, we established the selective quantification of inorganic mercury in biological samples following complete removal of MeHg by toluene extraction.[7] In principle, if MeHg extracted by toluene can be transferred to an aqueous medium, its analysis must also be possible by AAS. Here, we attempted to analyze MeHg levels in biological samples using the AAS instrument.

MATERIALS AND METHODS

Chemicals —— MeHg chloride and L-cysteine were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). MeHg chloride was used in the experiment without further purification, since contamination of inorganic Hg was confirmed to be less than 0.05%. MeHg chloride was dissolved in phosphate buffered saline (pH 7.4) as its L-cysteine conjugate, and stored at –80°C until use. Glutathione (reduced form) was the product of Wako Pure Chemical Industry Ltd. (Osaka, Japan).

Recovery of MeHg —— Cerebrum, liver and kidney were obtained from male Wistar rats (CLEA Japan, Osaka, Japan, aged 10-weeks). The tissue samples were homogenized (10%, w/v) in distilled water using a Polytron homogenizer (Kinematica GmbH, Littau, Switzerland). MeHg-L-cysteine was added to each homogenate at 2.5 μM (0.5 ppm as Hg) concentration. 0.25 ml of the homogenate was acidified with 6 M HCl (0.1 ml) in a 2-ml screw-capped polypropylene tube, and toluene (1 ml) and 4 zirconium particles (2-mm diameter) were added. The mixture was vigorously mixed at 3000 rpm for 2 min using a micro homogenizing system MS-100 (Tomy Digital Biology Co., Ltd., Tokyo, Japan), then centrifuged at 12000 rpm for 1 min. 0.5 ml of the toluene solution at the upper phase was transferred to a 1.5-ml polypropylene tube, and 0.25 ml of 5 mM glutathione in 100 mM sodium phosphate buffer (1 mM EDTA, pH 7.5) was added. The mixture was shaken for 5 min using a microtube mixer MT-360 (Tomy Digital Biology Co., Ltd., Tokyo, Japan) to extract MeHg by the aqueous phase as its glutathione conjugate. After centrifuging at 12000 rpm for 1 min, toluene was removed by aspiration; an insoluble material at the interface, if extant, remained. The aqueous phase was mixed with petroleum ether (0.5 ml) using a Vortex mixer for 10 sec, and centrifuged at 12000 rpm for 1 min, then the organic phase was removed by aspiration. The residual petroleum ether was removed completely under an air stream of aspiration. The sample solution thus prepared was diluted by 2 times from the initial homogenate, and contained exclusively MeHg. The mercury level in the final sample was determined together with the initial homogenate by the oxygen combustion-gold amalgamation method[6] using an atomic absorption mercury detector MD-1 (Nippon Instruments, Co. Ltd., Osaka, Japan). The recovery of MeHg added was calculated from mercury levels in both sample solutions.

Selective Quantification of Total, Methyl and Inorganic Mercury in Control and MeHg-Treated Rat Tissues —— Eight of 12 male Wistar rats (CLEA Japan, Osaka, Japan, age 10 weeks) were orally injected MeHg chloride (5 mg/kg), and brain, liver and kidney were removed on day 1 and 8 after injection, following ether anesthesia and saline perfusion from the heart. The tissue samples were homogenized (20%, w/v for control and 10%, w/v for MeHg-treated group) using a Polytron homogenizer. The homogenates were used in total mercury analysis. The MeHg sample was prepared as described above. Samples for inorganic mercury analysis were prepared according to the previous protocol[7] with some modification. Briefly, 0.25 ml aliquot of each homogenate was acidified with 6 M HCl (0.1 ml) and mixed with toluene (1 ml) in a 2-ml screw-capped tube with 4 zirconium particles (2-mm diameter) using the micro homogenizing system, and centrifuged at 12000 rpm for 1 min. After removing the upper phase, MeHg in the homogenate was completely removed by an additional 4 toluene extractions. The aqueous phase thus prepared and containing exclusively inorganic mercury was washed once with petroleum ether (1 ml) to remove residual toluene, and neutralized with 4 M NaOH (0.15 ml). Mercury concentration in this prepared sample was analyzed as the inorganic mercury level.

RESULTS AND DISCUSSION

Mercury analysis by the oxygen combustion method gives results as total mercury levels, since the combustion process converts both methyl and inorganic mercury in the sample to mercury vapor. Accordingly, pretreatment of analytical samples is necessary for a selective quantification of mercurial species. We previously reported a selective quantification of inorganic mercury in MeHg-treated rat tissues that contained both methyl and inorganic mercury.[7] The inorganic mercury samples were prepared from the tissue homogenate by 4 to 5 repeated toluene extractions to remove existing MeHg. Since the toluene solution in the first extraction contained the most MeHg of the sample, we supposed the MeHg level might be estimated, if it could be effectively transferred to an aqueous medium. Back extraction of MeHg in organic phase is possible using a thiol solution such as cysteine and glutathione in the basic medium.[2] MeHg conjugates of these two thiol compounds have similar stability constants, and the pKa values are 15.9 and 15.7 for glutathione and cysteine conjugates, respectively.[8] We employed here glutathione in a slightly basic phosphate buffer (pH 7.5), since it was more stable in the basic medium due to lower ionic strength than cysteine.[9] In the preliminary experiment, we found no difference between these two thiol compounds in the MeHg extraction from the toluene solution.

Since all the procedures were carried out using microtubes, micro homogenizing system and micro centrifuge, time consumption in each step was as short as a few minutes. Accordingly, 30 min was

Table 1. Recovery of MeHg Added to Rat Tissue Homogenate

Tissue	Brain	Liver	Kidney
Recovery of MeHg (%)	86.7 ± 1.4	83.6 ± 1.2	86.1 ± 1.1

To 10% homogenate of rat tissues MeHg-L-cysteine was added at 2.5 μM concentration. MeHg added was extracted by toluene, following acidification with HCl, and back extracted in glutathione solution. Rate of mercury levels in the final solution to the original homogenate was shown.

Table 2. Methyl, Inorganic and Total Mercury Levels in MeHg-Treated and Control Rat Tissues

Tissue	MeHg (A)	Inorganic Hg (B)	Total Hg (C)	% of MeHg	(A + B)/C
1 day					
Brain	0.712 ± 0.026	0.029 ± 0.013	0.735 ± 0.03	96.9 ± 1.8	1.01
Liver	2.97 ± 0.15	0.16 ± 0.02	3.18 ± 0.15	93.4 ± 0.8	0.98
Kidney	13.4 ± 1.5	1.6 ± 0.1	15.9 ± 1.5	84.4 ± 2.6	0.95
8 day					
Brain	0.959 ± 0.044	0.141 ± 0.035	1.014 ± 0.051	94.7 ± 5.1	1.08
Liver	1.96 ± 0.34	0.30 ± 0.02	2.32 ± 0.3	84.1 ± 4.4	0.97
Kidney	10.5 ± 0.8	14.4 ± 3.4	25.3 ± 3	41.8 ± 4.2	0.98
Control					
Brain	10.2 ± 0.9	12.6 ± 1.9	22.0 ± 2.5	46.6 ± 4.8	1.04
Liver	12.9 ± 2.2	22.2 ± 7.2	35.1 ± 10.7	38.2 ± 5.9	1.01
Kidney	32.5 ± 3.2	63.7 ± 3.9	100.3 ± 7.2	32.7 ± 5.4	0.96

Methyl, inorganic and total mercury levels in rat tissues were determined at 1 and 8 days after MeHg (20 μmol/kg, po) administration. Each mercury level represents mean ± S.D. obtained from 4 rats as μg Hg/g tissue for MeHg-treated rats and ng Hg/g tissue for control rats.

sufficient to prepare the MeHg samples using 12 tubes, which was the capacity of the micro centrifuge used. A micro homogenizing system employed here for the toluene extraction had been developed for DNA sample preparation by homogenizing animal and plant tissues in microtubes using glass or zirconium particles. The instrument was considered to be more effective to extract MeHg, especially from the tissue homogenate that sometimes formed partial coagulate when acidified. The micro homogenizing system using zirconium (or glass) particles changed the coagulated mixture to a fine emulsion, probably leading to an effective MeHg extraction. In the toluene extraction a huge amount of amorphous material formed at the interface often makes for a poor separation.[5] The centrifugation at 12000 rpm for 1 min employed here was sufficient to precipitate the amorphous material to a flat shape. No difficulty was found to take the half volume of toluene at the upper phase.

Recovery of MeHg added to rat tissue homogenates at 2.5 μM concentration was around 85% after toluene extraction and back extraction to the glutathione solution (Table 1). Since the rate of MeHg recovery was unchanged between 0.5 and 25 μM addition (data not shown), the method would be applicable for a wide range of mercury concentrations. When 0.1 M NaOH was used as an aqueous medium[2] a considerable amount of amorphous material was often formed at the interface, and it made removal of toluene phase difficult. However, the formation of the amorphous material was drastically suppressed by using the basic phosphate buffer. Furthermore, no significant difference was observed in the MeHg recovery between two aqueous media (data not shown).

Methyl, inorganic and total mercury concentrations in brain, liver and kidney of rats sacrificed 1 and 8 days after MeHg administration are summarized in Table 2 with those of control rats without MeHg injection. MeHg values were calculated using a recovery factor of 0.85. Comparing the MeHg concentration in the first and second toluene extracts using gas chromatography, about 90% of MeHg was found to be extracted by a single toluene partitioning (data not shown). Accordingly, five repeated extractions by toluene were considered to be sufficient for a complete (> 99.99%) removal of coexisting MeHg. The sum of methyl and inorganic mercury values fitted well with the total mercury in all

tissues, including control rat tissues. The present method would be useful to estimate the MeHg level in animal tissues as low as ng/g (ppb) levels in control rats.

For validation, methyl, inorganic and total mercury levels of DORM-2 (a reference material from dogfish, National Research Council, Canada) were analyzed by the present method. The certified values for total and methyl mercury are 4.64 ± 0.26 and 4.47 ± 0.32 μg/g, respectively. The values obtained by the present method without the MeHg recovery factor were 4.51 ± 0.14, 3.91 ± 0.03 μg/g and 0.34 ± 0.02 μg/g for the total, methyl and inorganic mercury, respectively. The sum of methyl and inorganic mercury values yielded about 94% of the total mercury value due to a somewhat lower MeHg value. Calculations using the MeHg recovery factor of 0.90 resulted in 4.35 ± 0.03 μg/g, that well fitted the certified value. The sum of methyl and inorganic mercury also fitted the total mercury amount. The different recoveries of MeHg between rat tissue and DORM-2 might be due to kind, rat tissue and fish meat, or form, wet tissue and lyophilized powder, of the samples. When the method is applied to other tissue samples, such as blood, wet fish or plants, MeHg recovery may have to be confirmed prior to analysis.

Strictly speaking, the present method affords a quantification of toluene-extractable organic mercury, not limited to MeHg. However, since MeHg is the exclusive organic mercury in the natural environment, the data obtained here would reflect that of MeHg. Although a fairly constant recovery of MeHg of around 85% was obtained in the rat tissues, the residual portion might remain in the original homogenate and/or in the toluene extract. Some further modification to increase the recovery might be necessary to improve reliability of the method. Nevertheless, it would be useful to estimate, at least roughly, the MeHg content using the same instrument as total mercury analysis.

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