QUANTITATIVE EVALUATION OF ENVIRONMENTAL FACTORS INFLUENCING THE DYNAMICS OF MERCURY IN THE AQUATIC SYSTEMS

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Abstract

Highly sensitive and systematic methods for determining total mercury and methylmercury in various biological and environmental materials have been established to study and evaluate the environmental factors influencing the dynamics of mercury in aquatic system. For the analysis of total mercury, a biological or sediment sample is digested in a 50ml thick walled digestion flask using HNO_3–$HClO_4$–H_2SO_4 (1:1:5) mixture by heating at 200±5°C on a hotplate for 30 minutes. In the analysis of water, the sample is treated first with $KMnO_4$ and H_2SO_4 and then extracted with dithizone in toluene. The extract is evaporated to dryness and digested in the same manner as described above. After cooling, the digested sample is filled up to the 50 ml mark with mercury-free water. The analysis of mercury in the sample solution is done by cold-vapor atomic absorption spectrometry using a semi-automated system recently developed in our laboratory. With this system, sensitivity and accuracy are substantially improved and the determination of the sample is completed within one minute. The detection limit for this method is 0.5 ng Hg in the sample solution.

Analytical procedure for methylmercury in biological samples consists of: (1) alkaline digestion with 1N KOH in ethanol; (2) washing out fatty materials with hexane after slightly acidified with 1N HCl; (3) extraction with dithizone in toluene ; (4) clean-up with Na_2S; (5) re-extraction with dithizone in toluene; and (6) measurement of methylmercury by ECD-gas chromatography. For methylmercury in sediment or water samples, the sediment is treated with 1N KOH in ethanol, whereas the water sample is treated with $KMnO_4$ and H_2SO_4. After these pretreatments, methylmercury is extracted with dithizone in toluene and then followed by clean-up with Na_2S, re-extraction with dithizone in toluene and measurement of methylmercury in the same way as described above. The detection limit of these procedures is around 5 ng/g in a 0.5 g sample on a wet weight basis.

1. INTRODUCTION

Our knowledge of mercury cycling in the aquatic systems which involve transformation, distribution, and accumulation has improved significantly in recent years because of the development of highly sensitive and systematic analytical techniques. The improvement of analytical methods and renewed interest in environmental mercury research triggered by the discovery of high mercury levels in fish from pristine remote lakes in US, Canada and Scandinavia (Stephens, 1995; Andersson, et al, 1995) and the increase of mercury pollution from gold mining activities in developing countries, has made it necessary to re-evaluate the existing data on the biogeochemistry of mercury. The past and present health and environmental impacts of mercury of natural and anthropogenic origin in different countries continue to motivate studies on the environmental cycle of mercury.

A better understanding of mercury distribution in aquatic systems not only requires knowledge of total mercury, but also the amount of mercury that will eventually be produced in methylated form which is much more available for bioaccumulation. The conversion of inorganic mercury into methylmercury has long been recognized as a critical step in the environmental behavior of this metal, however, the mechanism of the synthesis of

methylmercury is not very well understood. Moreover, it is important to know the chemical forms of mercury in the sediment, since organic mercury is water-soluble and easily taken up by aquatic organisms to be biomagnified up through the aquatic food chain. The current understanding of the accumulation of methylmercury in the marine food chain is limited. The determination of the methylation sites and processes and of methylmercury pathways and fluxes in the environment are therefore essential in the understanding and prediction of mercury migration into the environment since a major source of human exposure to methylmercury is through the consumption of fish and fish products.

Previous studies have used radiochemical methods to investigate the speciation and distribution of mercury in aquatic systems (Akagi, et.al, 1979). Inorganic mercury and methyl mercury in sediment and water were extracted in dithizone-benzene and measured after separation by thin layer chromatography (Guimaraes et al., 2000). Other researchers have used analytical methods based on the Westöö (1968) procedure to extract mercury from environmental samples followed by the analysis of methylmercury by gas chromatography with electron capture detection (GC-ECD). In most of these and earlier studies, methylmercury extraction efficiency and the results of the certified reference materials were not reported. Also the presence of interfering organic compounds reduced the accuracy of the methylmercury analyses. This limited the reliability of methylmercury data for evaluation of methylmercury production and partitioning in various compartments of aquatic systems. In the present study, highly precise and accurate analyses for T-Hg and Me-Hg have been used to constrain levels of MeHg production in laboratory experiments involving sediment-water systems with or without fish.

2. MATERIALS AND METHODS

Total mercury (T-Hg) and methylmercury (MeHg) in fish, sediment and water samples is extracted and analyzed using reliable, highly sensitive and accurate techniques developed at the National Institute for Minamata Disease (NIMD, Japan) by Akagi and Nishimura (1991) and later modified by Akagi (1997) and Ikingura and Akagi(1999).

2.1. Analytical Procedure for Total Mercury

The measurement of T-Hg is based on cold vapor absorption spectrometry. The principle of this method involves reducing ionic Hg^{2+} in sample solutions using tin (II) chloride to generate metallic Hg. The sample is then aerated and the Hg vapor generated is introduced into an absorption cell and the absorption measured at 253.7nm. A semi-automated mercury analyzer (Hg-5000, Sanso Seisakusho Co. Ltd, Japan) is used for mercury detection and measurement.

2.1.1. Sample Preparation and Treatment

All glassware should be cleaned with acidic KMnO4 solution (0.5% $KMnO_4$ in 1 N H_2SO_4) and Hg-free water prior to sample preparation.

(i) Hg standard solution
Dissolve 12.5mg of CH_3HgCl in toluene to make a final volume of 100ml. Dilute this solution 100 times using toluene to prepare a concentration of 1 ppm methylmercury chloride in toluene. 1ml of this solution contains 1.0µg of Hg (1000ng Hg).
Methylmercury-cysteine solution: Mix 0.5ml of 1ppm methylmercury standard solution in toluene and 5ml of 0.1% L-cysteine in 0.1N NaOH solution into a 10ml pyrex glass test tube.

Shake for 3 minutes and the centrifuge at 1000rpm for 3 minutes. Discard the organic phase (top layer). Seal the tube and store the Hg standard solution in a cool, dark place. 1ml of this solution contains 0.1μg (100ng) of Hg. Prepare a fresh solution every month.

(ii) Sediment and Fish Samples
Accurately weigh out the sample (0.5g or less wet weight) into a digestion flask. Add 1ml of distilled H_2O, 2ml of HNO_3 – $HClO_4$ (1:1) and 5ml of H_2SO_4. Then add glassfibre chips to prevent violent boiling on heating. Wipe the flask to ensure that chemicals were not spilled onto the surface. Heat the flask on a hot plate at 200 ±5°C for 30 minutes to digest the sample. Allow the flask to cool and then add H_2O to obtain a fixed volume, usually 50 ml. Use the resulting solution for Hg analysis. Prepare a standard solution by measuring 1ml (corresponding to 0.10μg of Hg) of Me-Hg-cysteine solution (0.10μg Hg/ml). Add the reagents to the standard and follow the same digestion procedure as described above for the preparation of the sample solution. For the blank, add the reagents to an empty flask and follow the digestion procedure.

(iii) Water samples
Transfer 1L of water sample into a separatory funnel. Add 5ml of 20N H_2SO_4 and 2ml of 0.5% $KMnO_4$ solution. Mix and let stand for 5 minutes. Neutralize using 10ml of 10N NaOH and mix with 2ml of 10% hydroxylamine-HCl solution. Stand for 20 minutes. Neutralize with 2ml of 10% EDTA and then add 5ml of 0.01% dithizone solution. Mix and stand to allow complete separation. Discard the aqueous layer (lower phase). Transfer 3ml of the organic layer to a sample digestion flask. Using a rotary evaporator, immerse the flask in water bath at 60°C and evaporate to dryness. Digest the residue by following the procedure indicated above for fish/sediment samples. Prepare a separate 1L Hg free water as the blank sample and another 1L Hg-free water spiked with 200μl (corresponding to 20ng as Hg) of Me-Hg cysteine solution (0.10μgHg/ml) as the standard. Follow the extraction and digestion procedures described above and use the resulting solution for T-Hg analysis.

2.1.2. Determination of Mercury Concentration

The total volume of the solution needed for T-Hg analysis is 10 ml. Before analyzing Xml of blank sample, standard sample or the actual sample solution for T-Hg, attach a calibrated dispenser to the Hg-5000 semiautomatic analyzer (Sanso Seisakusho Co. Ltd, Japan) ready to dispense a known volume of distilled water. Gently add Xml (solution + water, maximum of 10ml) of the sample solution to a reaction vessel, insert a stopper to close the vessel and then inject 1 ml of tin (II) chloride solution from the accessory syringe. Press the start button of the analyzer. The diaphragm pump will operate and the generated Hg vapor will be circulated through the four-way cock between reaction vessel and acidic gas collection bottle. During this circulation, acidic gases leaving the sample solution are removed by an acidic gas trap containing 5 N NaOH. After 30 seconds, when the concentration of Hg vapor has reached equilibrium, the vapor is introduced into the absorption cell automatically by turning the 4-way stop cock by 90°. When the maximum peak height has been recorded, the sample is discarded from the reaction vessel and purged with air to remove the residual Hg vapor. After purging, the valve controller is reset, by pressing the reset button, to start the next measurement.

After analysis, mercury concentration in the samples is calculated from mercury absorbance peak heights recorded on the chart by the plotter. The peaks heights (mm) for the blank solution (Pbl), mercury standard (Pstd), and actual sample (Psp) are used to calculate mercury concentrations using the following formulae.

a) Fish and sediment

T-Hg concentration in the sample (mg/g):

$= 0.1$ mg x $(P_{sp} - P_{bl})/(P_{std} - P_{bl})$ x dilution factor x 1/sample weight (g)

b) Water samples

T-Hg concentration in the sample (ng/L):

$= 20$ng x $(P_{sp} - P_{bl})/(P_{std} - P_{bl})$ x dilution factor x 1/sample volume (L)

2.2. Analytical Procedure for Methyl Mercury

For Me-Hg and other compounds, Gas Chromatograph with electron capture detector (GC-ECD) is often used for determination and measurement. This method provides good separation and superior sensitivity for the determination of halogenated methylmercury compounds. Conventionally, it has been widely used for the quantification of MeHg in various types of biological and environmental samples.

2.2.1. Methylmercury Extraction

All glassware for processing the samples should be washed with toluene just before use in order to remove any organic contaminants.

(i) Fish

Weigh Xg of homogenized sample (0.5g or less as wet weight, approximately 0.1g for dry samples) in a 50ml screw-capped conical centrifuge tube. Add 10ml of 1N KOH – C_2H_5OH (1:1) solution. Seal tightly and heat in a 100°C isothermal bath for 1 hour with occasional mixing. Allow to cool. Add 10ml of 1N HCl and 5ml of hexane and shake for 3 minutes (to remove fats) using a reciproshaker. Centrifuge at 2000rpm for 3 minutes then suck off and discard the hexane (upper layer). Add 2ml of 20% EDTA and 10ml of purified 0.01% dithizone solution. Shake to extract the methylmercury as dithizonate (complex) in the toluene layer (upper layer). Centrifuge at 2000rpm for 3 minutes then suck off and discard the lower layer (aqueous layer).

Add 5ml of 1N NaOH to the toluene layer, shake (to remove excess dithizone) and centrifuge at 2000rpm for 3 minutes. Suck off and discard the lower layer (aqueous layer). Repeat the above clean-up procedure. Let the solution settle for a while, remove the lower layer, and centrifuge again at 2000rpm for 3 minutes to obtain a clear toluene layer. Transfer a fixed volume of the toluene layer (normally 4ml) to a 10ml glass-stoppered conical centrifuge tube. Add 2ml of alkaline Na_2S solution, and shake to back-extract the MeHg into the aqueous layer. Centrifuge at 1000rpm for 3 minutes then suck off and discard the upper toluene layer. Wash the aqueous layer with 2ml of toluene, shake for 2-3 minutes and centrifuge at 1000rpm for 3 minutes. Suck off and discard the toluene layer (upper layer). Acidify with 1N HCl (3-4 drops,). Bubble the solution with N_2 gas gently at a flow rate of 50ml/min for 3 minutes. Re-extract the MeHg with 2 ml of Walpole's buffer solution and purified 0.01% dithizone solution (0.2 ~ 1.0ml, normally 0.5ml). Shake for 2-3 minutes and centrifuge at 1000rpm for 3 minutes, suck off and discard the lower aqueous layer. Add 3ml of 1N NaOH to the toluene layer and shake to remove excess dithizone. Let the solution settle, then suck off and remove the aqueous layer (lower layer). Centrifuge at 1000rpm for 3 minutes. Suck off and discard the lower layer as much as possible. Acidify with 2 drops of 1N HCl. Vortex mix and use the resulting solution as the sample for GC-ECD methylmercury analysis. Perform the sample solution preparation protocol for the standard using 200µl (corresponding to 0.020µg of Hg) of MeHg -cysteine. Use the resulting extract as the MeHg standard.

(ii) Sediment
The procedure for MeHg analysis in sediment involves digesting the sample with 1N KOH solution in ethanol (1N KOH-EtOH). Slight acidification with 1 N HCl, and extraction of MeHg by 10 ml of purified 0.01% dithizone-toluene solution. This is followed by the clean-up of the Dz-Tol extract and the analysis of MeHg by GC-ECD. In the clean-up of the Dz-Tol extract of a sediment sample, the extract is passed through a florisil column (0.5g florisil, topped with 0.5g Na_2SO_4) and washed twice with I N NaOH before the extract is subjected to back extraction with Na_2S following the procedure outlined above for fish samples. The clean-up procedure removes the organic materials in the sample that may interfere with MeHg analysis by GC-ECD.

(iii) Water
The extraction procedure for MeHg in water is the same as that for T-Hg extraction described in part 2.1.1 (iii). After extraction, the dithizone-toulene extract of the water sample is carried through the same clean-up procedure as describe for the fish samples, followed by MeHg analysis by GC-ECD. Perform the sample solution preparation protocol for the water MeHg standard using 10μl (corresponding to 1ng of Hg) of MeHg-cysteine. Use the resulting extract as the MeHg standard.

2.2.2. Methylmercury Detection and Measurement

Gas Chromatograph
A gas chromatograph with an electron capture detector (GC-ECD) is used for methylmercury determination and measurement. The gas chromatography conditions are set as follows:
Column: Use a glass column (3mm x 0.75~1.0m) packed with Hg-20A-Uniport HP (GL Science, 60 ~ 80 mesh) or 10% KOCL-Hg-ChromosorbW (AW/DMCS, Yanaco, 60 ~ 80 mesh). At the injection port, pack 2 ~ 3 centimeter of NaCl previously heated at 500°C for 2 ~ 3 hours
Temperature: Column oven: 140 ~ 160°C, injection port: 180°C, Detector oven: 200°C
Carrier gas: N_2, 30 ~ 40ml/min

Pre-Analytical Run
Before analzing the samples, perform a trial run on toluene by gas chromatography to ensure that no other peak co-elutes with the expected retention time for MeHg. Then inject separately a fixed volume (normally 5μl) of the blank, standard sample, and actual sample into the gas chromatograph. Label the peak heights thus obtained as P_{bl}, P_{std}, and P_{sp}, respectively. The concentration of methylmercury is calculated from the peak heights using the following formulae.

Sediment
MeHg concentration in sample (μg/g) = 0.020(μg) x $(P_{sp}-P_{bl})/(P_{std}-P_{bl})$ x dilution factor x 1/sample weight (X g, dry wt)

Fish
MeHg concentration in fish sample (ng Hg/g) = 100(ng) x $(P_{sp}-P_{bl})/(P_{std}-P_{bl})$ x dilution factor x 1/sample weight (X g, wet wt)

Water
MeHg concentration in water samples (ng/L) = 1(ng) x $(P_{sp}-P_{bl})/(P_{std}-P_{bl})$ x dilution factor x 1/ sample volume (L)

3. DISCUSSION

In order to correctly elucidate and evaluate the degree of Hg contamination, reliable analysis of data based on proper monitoring methods is required as follows: (1) proper choice of sample; (2) appropriate sample collection, storage, and transport; (3) sample preparation techniques; (4) analytical methods/procedures (5) experienced/trained staff. In addition, when conducting analyses, regular housekeeping must be maintained in order to keep the laboratory clean and glasswares, tools and containers free from contamination. Aside from this, adequate ventilation and personal protective equipment should be provided including facilities to handle such chemicals.

Methods for T-Hg measurement include absorption spectrophotometry (dithizone colorimetry), cold vapor atomic absorption spectrometry (CVAAS), fluorescence spectrometry; and neutron activation analysis.

In absorption spectrophotometry, dithizone forms a chelate with the metal ions and produces a colored organic solution. The intensity of the color depends upon the Hg concentration. Although the method has been used historically due to the simplicity of the procedures, its use has declined greatly with the introduction of the highly sensitive atomic absorption spectrometry in the 1960s. Atomic absorption spectrometry uses the property of metallic Hg to volatilize into its atomic form. The Hg vapor is introduced into an absorption cell and the absorption measured at 253.7nm.

In neutron activation analysis (NAA), the sample is irradiated with neutrons and the gamma radiation emitted by ^{197}Hg formed, is then measured by spectrometry. Although NAA is highly sensitive and requires minimal sample preparation, it is not frequently used due to its high cost, the need for a nuclear reactor and an expensive counting apparatus including the safety requirements for handling radioactive material.

The cold vapor atomic absorption spectrometry (CVAAS) is a much more sensitive method as compared with the conventional flame atomic absorption spectrometry. Aside from this, other advantage includes its ability to measure samples using a UV spectrophotometer with a simple Hg lamp. The first method which is currently the most commonly used, involves sample digestion with strong acids followed by reduction and vaporization of Hg while the other method involves heating and vaporization through direct combustion of the sample to obtain metallic Hg. For accurate analysis of T-Hg, complete digestion of organic materials in the samples is essential. A number of wet digestion procedures have been proposed and used but most of them involve time consuming operations and require considerable number of reagents and careful handling during digestion. Various combinations of acids and oxidizing agents have been used since a major problem in the sample preparation procedure is mercury loss during the digestion process. Based on studies conducted, the presence of oxidizing agents ($HClO_4$ or 5% $KMnO_4$) prevents the loss of Hg completely even under extreme heating conditions. Of these two oxidizing agents, $HClO_4$ was selected because the use of $KMnO_4$ will require another reagent. In normal H_2SO_4 – HNO_3 digestion, mercury may vaporize during the reaction process. However, in the presence of an oxidizing agent which is incorporated beforehand, Hg vaporization under severe heating conditions can be completely prevented. The use of a long neck (10cm or more) thick walled flask as the digestion container will prevent Hg loss even with heating at 200 ~ 250°C.

In water samples the concentration of Hg is extremely low and its measurement requires some preconcentration. Of the methods reported, the dithizone extraction technique described by

Chau and Saitoh (1970) was reported to be applicable for this purpose. As a result, pretreatment with H_2SO_4 and $KMnO_4$ was found to be essential to obtain satisfactory recoveries. The reason for this is presumably due to the release of ionized Hg compounds from binding sites on organic material and particulate matter in water samples by oxidation. When preparing water samples for analysis, the addition of hydroxylamine-HCl neutralizes the strong oxidizing property of $KMnO_4$ and the addition of EDTA prevents the interference caused by other metals in the sample. Both are therefore added to protect dithizone from oxidation and unnecessary cross reactions with other metal ions. In a fish sample test, T-Hg detected remained constant during a 10-60 minute heating time indicating that digestion was complete within 10 minutes.
The widely used benzene extraction developed by Westoo (1966) for MeHg analysis of environmental samples such as fish and sediment resulted in poor extraction efficiency and requires a longer time for extraction. Previously, a highly sensitive radiochemical technique was developed involving extraction of Hg with dithizone in benzene (Dz-Bz) and separation by thin layer chromatography followed by gamma counting of each fraction for 203 Hg.

In addition, a highly precise and systematic analytical method has been developed for methylmercury determination which is capable of analyzing various samples. Pretreatment of biological samples by KOH solution in C_2H_5OH has been found to be very effective in extracting Hg with dithizone-benzene after acidification. Meantime, alkaline digestion is advantageous for analysis of MeHg particularly in biological samples since the digests are clear and do not form any emulsion on initial solvent extraction. This can be attributed to the fact that proteinaceous materials are broken down in the sample matrices during digestion. This alkaline treatment can also be applicable to the pretreatment of sediment samples since it often contains humic substances and sulfide ions having a great affinity for Hg compounds. The humic substances are soluble in alkaline solution and thus MeHg is released and sulfide ions can be removed by N_2 bubbling. Westoo (1966) has reported gas chromatographic methods for estimating Me-Hg in fish and showed that MeHg dithizonate has the same retention time for chloride or bromide homologues. This finding strongly suggests that the determination of MeHg in various samples by the combination of dithizone extraction and gas chromatographic separation is possible. This was validated by gas chromatography-mass spectrometry method indicating the immediate conversion of methylmercuric dithizonate into its chloride form. Freshly prepared and purified dithizone solution should be used for the extraction since diphenylthiocarbadiazone, the oxidized form of dithizone, may interfere with the determination of MeHg in the GC analysis. Furthermore, toluene is preferred over benzene, for health and safety reasons.

The methods described in sections 2.1 and 2.2 in this paper have been repeatedly verified as to their accuracy and precision through inter-laboratory comparison (Matsuo et al., 1989, Kehrig et al., 1998) and the analysis of certified reference materials (e.g TORT) for T-Hg and MeHg. Also these methods have been used in the certification of the reference materials from the International Atomic Energy Agency (IAEA Horvat et.al., 1997). The detection limits are 5 ng/g for T-Hg and 5 ng/g for Me-Hg in biological and sediment samples and 0.5 ng/L for T-Hg and 0.1 ng/L for Me-Hg in water samples.
Whatever methods are used, the implementation of quality assurance and quality control procedures should be undertaken to check analytical measurements regularly. One good practice is the regular use of the appropriate reference materials such as the use of IAEA-085, IAEA-086, and IAEA-142 as standard materials. Currently, a number of standard reference materials are commercially available through the National Institute of Standards and Technology (NIST), the International Atomic Energy Agency (IAEA), and the National Research Council of Canada (NRCC).

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