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Quantitative detection of organic mercury in whole blood using derivatization and gas chromatography-negative chemical ionization-mass spectrometry

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Abstract

The Minamata disease, first identified in Japan in the 1950s, is caused by severe methylmercury (MeHg) poisoning. To prevent the development of this disease, routine evaluation of MeHg levels in blood samples is crucial. The purpose of this research was to explore the use of derivatization and capillary gas chromatography-negative chemical ionization-mass spectrometry (GC-NCI-MS) for the quantitative detection of both organic and inorganic mercury in blood samples. Alkyl mercury in standard solutions was extracted as halide salts in toluene with hydrohalic acid. Fat contents in whole blood samples were removed by methyl isobutyl ketone and hexane using a cysteine/alkaline solution and then organic mercury was extracted as a bromide complex using toluene and cupper chloride solution. The linearity of the response ratio *vs.* concentration curves (R^2) was 0.987 for methylmercury bromide and 0.990 for ethylmercury (EtHg) bromide, over the calibration range of 0.02 ng/mL to 20 ng/mL. The recovery of MeHg and EtHg was 67.1% and 49.3%, respectively. The concentrations of MeHg in whole blood samples determined using GC with an electron capture detector agreed with those determined using GC-NCI-MS, with a correlation coefficient of 0.923. The mean concentration of MeHg in a certified reference material (NMIJ CRM 7402-a) determined using GC-NCI-MS was 0.64 μg/g, comparable with the certified value of 0.58 μg/g. Our study demonstrates a simple and low-cost approach for analyzing mercury in biological samples, although further optimization is required given the relatively low recovery and the concern about the toxicity of methyl isobutyl

ketone.

Keywords: Organic mercury, blood, derivatization, gas chromatography, negative chemical ionization, mass spectrometry

INTRODUCTION

At the beginning of 1950, the catastrophic discharge of extremely high levels of methylmercury (MeHg) detected in Minamata Bay, Kyushu Island, Japan caught the public's attention. This level of MeHg contamination resulted in marine pollution and residents presenting with sensory and motor symptoms, primarily through ingestion of contaminated seafood from the bay[1]. This neurological disorder was later called the Minamata disease. From the beginning of the Minamata Bay outbreak to the year 2000, the number of affected residents reached 2264, with 200,000 inhabitants suspected of MeHg poisoning[1].

Although Japan has adopted various measures to rectify mercury pollution, the Ministry of the Environment reported that in 2016, a total of 16.78 tons of mercury was discharged into the environment, of which 16 tons were emitted into the atmosphere, 0.58 tons into the ground, and 0.20 tons into water[2]. According to the 2013 Global Mercury Assessment report, mercury was emitted into the environment at 5500-8900 tons per year, of which 30% was anthropogenic (e.g., coal burning and gold mining), 10% was naturally generated, and 60% was re-emitted[3]. Therefore, reducing anthropogenic emissions of mercury is crucial to reducing the amount of mercury circulating in the environment.

Since the Minamata Bay outbreak, various analytical methods have been developed to evaluate the levels of mercury compounds in the environment and biological samples[4]. At present, cold vapor atomic absorption spectroscopy is primarily used to determine the total mercury content in samples[4-5]. However, this method is limited to total mercury. Because the toxicity of inorganic mercury and organic mercury in humans is different[6], it is necessary to determine the contents of the two species. Packed column gas chromatography-electron capture detector (GC-ECD) has been used to determine the organic mercury content separately because of its high detection sensitivity for organic mercury chloride[7]. However, the measurement steps of this method are very complicated owing to the limited selectivity of ECD. In particular, the interference of co-extracted halogen-containing compounds necessitates sophisticated sample extraction and cleaning steps. Other techniques have emerged for the analysis of mercury species with high sensitivity and selectivity, such as gas chromatography-atomic fluorescence spectrometry (GC-AFS)[8] and gas chromatography-inductively coupled plasma-mass spectrometry (GC-ICP-MS)[9]. Nevertheless, for routine analysis, a simple, low-cost, and readily available method is still required.

In recent years, an analytical method using alkylation and phenylation by sodium tetraalkylborate and tetraphenylborate salts has been developed for capillary gas chromatography-mass spectrometry (GC-MS)[8,10]. Capillary GC-MS is a conventional method widely applied for structural confirmation and routine analysis of various toxic contaminants in environmental matrices and biota. The method employs electron ionization-mass spectrometry (EI-MS), which is less sensitive than ECD. However, there are different ionization methods for MS, one of which is negative chemical ionization (NCI), which facilitates more specific and sensitive detection than EI. In particular, NCI mass spectra typically contain an abundance of quasi-molecular ions with few fragment ions[11]. MS with NCI may provide higher sensitivity and selectivity than ECD. However, to date, few studies have used GC-NCI-MS for the analysis of mercury species.

The purpose of this research was to explore the use of a derivatization method and GC-NCI-MS for the quantitative detection of both organic and inorganic mercury in blood samples.

METHOD

Reagents

Standard solutions of organic mercury [MeHg and ethyl Hg (EtHg) in benzene, 10 μg/mL] and inorganic mercury (Hg^{2+}, 10 μg/mL) were obtained from Wako Pure Chemicals (Osaka, Japan). Sodium tetraphenylborate, sodium tetrakis [3,5-bis(trifluoromethyl)phenyl] borate ($BArF_{24}$) and lithium tetrakis (pentafluorophenyl) borate ethyl ether complex ($BArF_{20}$) were purchased from Tokyo Chemical Industries (Tokyo, Japan). A certified reference material (CRM) for MeHg (cod fish tissue, NMIJ CRM 7402-a No.250) was obtained from the National Institute of Advanced Industrial Science and Technology (Tsukuba, Japan). 2,4,6-Trichloroanisole-d_3 (TCA-d_3) standard solution (Wako Pure Chemicals) was used as an internal standard (1 μg/mL toluene solution) because labeled MeHg was not readily available.

Analysis of standard solutions

Standard mercury solutions (MeHg and EtHg in benzene, 10 μg/mL) were treated with derivatizing reagents (tetraarylborates) and then analyzed using GC-EI-MS and GC-NCI-MS. First, 0.1 M acetate buffer (pH 5) and 0.1 mL of 2% $BArF_{24}$, $BArF_{20}$, or tetraphenylborate and 0.5 mL of toluene were combined. The standard solution was shaken for 30 s and centrifuged (830 *g*, 5 min). The water phase was removed, and the solution was washed with 0.5 mL of fresh water. The toluene phase was divided into a GC vial. Mercury halides were extracted using halide salts and also analyzed using EI and NCI. The standard solution was mixed with HBr (5 M, 0.1 mL), $CuCl_2$ (2 M, 0.05 mL), and toluene (0.5 mL) in sequence. The mixture was shaken for 30 s and centrifuged at 830 *g* for 5 min. The toluene phase (0.5 mL) was transferred to a GC vial for GC-MS analysis.

Sample preparation

Purification and extraction of inorganic and organic mercury were performed following the method of a previous study[12]. To extract mercury compounds from whole blood samples (0.5 mL for unknown concentration samples and 0.1 mL for known high concentration samples), 0.1% cysteine solution (0.1 mL) was added to 2-mL polypropylene tubes. Then, the solution was made up to 0.5 mL with distilled water. Methyl isobutyl ketone (MIBK, 0.5 mL) was added, and the mixture was shaken for 30 s and centrifuged at 830 *g* for 5 min to remove the fat contents. The upper MIBK layer was removed, and the remaining solubilized water solution was again washed with 0.5 mL of hexane. The hexane layer was discarded.

To extract organic mercury, solubilized water solutions were mixed with HBr (5 M, 0.1 mL), $CuCl_2$ (2 M, 0.05 mL), 10 μL of the internal standard solution (10 ng per sample), and 0.5 mL of toluene in sequence. The mixture was shaken for 30 s and centrifuged at 830 *g* for 5 min. The toluene phase (0.5 mL) was transferred to a GC vial for GC-MS analysis.

To derivatize inorganic mercury in the remaining solubilized water solution, 0.1 mL of 0.1 M acetate buffer (pH 5), 0.1 mL of 2% $BArF_{24}$, and 0.5 mL of toluene were combined. The solution was shaken for 30 s and centrifuged (830 *g* , 5 min). The water phase was removed, and the solution was washed with 0.5 mL of fresh water. The toluene phase was divided into a GC vial.

Whole blood samples

Whole blood samples from a specimen bank were used to analyze organic mercury by GC-ECD[13]. Samples were chosen according to their MeHg concentration, from high to low. They were originally collected from Japanese adults in Kyoto Prefecture. Written informed consent was obtained from individuals before blood

sampling. This analysis was approved by the Kyoto University Ethics Committee (approved no. R1478).

Instrumental conditions

Agilent 6890GC equipped with a DB-5MS (15 m long, 0.25 mm i.d., 0.10 μm film thickness) capillary column was used (Agilent Technologies, Santa Clara, CA). The injection volume was 1 μL, and the inlet temperature was maintained at 280 °C. Pulsed splitless injection (30 psi for 2 min) was used, and the vent line was opened at 2 min. Helium gas was used as the carrier gas at a flow rate of 1 mL/min. The oven temperature was maintained at 40 °C for 2 min and ramped at 5 °C/min to 130 °C, 2 °C/min to 150 °C, and 30 °C/min to 240 °C. The transfer line temperature was 280 °C. Mass spectra were obtained using Agilent 5973MSD. In the EI mode, the ion source temperature was 230 °C, the emission current was 35 μA, and the electron energy was 70 eV. In the NCI mode, the ion source temperature was 150 °C, the emission current was 50 μA, and the electron energy was 120 eV. Methane gas was used as the reagent gas at a flow rate of 2 mL/min.

Full-scan mass spectra from m/z 50 to 700 were used to analyze standard solutions. Selected ion monitoring (SIM) was chosen for quantitative and qualitative detection using NCI-MS. Specific ions of organic mercury bromides were monitored for quantification (m/z 281) and identification (m/z 283 and 279) in the NCI mode. Calibration curves of target analytes were made by spiking the standard mixture at 7 points (0.01 ng per sample to 10 ng per sample) with the internal standard (10 ng of TCA-d_3 per sample). The amount ratio was calculated from the response of organic mercury to that of the internal standard (10 ng TCA-d_3). Linear calibration curves were fitted with a weight of the inverse of concentration. Instrument detection limits were calculated from the relative standard deviation of 8 replicated extracts from standard solution multiplied by 3 at 0.03 ng/mL standard solutions. Blank experiments were conducted before and after sample analyses (n = 6), while no signals of organic mercury were detected. To evaluate the method detection limits in whole blood samples, whole blood of unexposed Wistar rat were used because human blood contains residual levels of methyl mercury more than the limits. Methyl and ethyl mercury concentrations in the rat blood were determined by the method of standard addition and were 0.1 ng/mL and < 0.12 ng/mL, respectively. The rat blood was spiked to 0.15 ng/mL MeHg and 0.15 ng/mL EtHg. Method detection limits were calculated from the relative standard deviation of 8 replicated samples and extracts from spiked rat blood samples multiplied by 3.

RESULTS AND DISCUSSION

Derivatization of standard solutions

Tetraaryl borate salts were employed in this study because the carbon attached to borate is part of a nucleophilic group containing a polarized boron-carbon bond. In contrast, divalent mercury compounds are electrophilic and susceptible to transmetalation[14] [Figure 1].

For phenylmercury compounds, fragment ions were observed in the EI mode, whereas no fragment ions were observed in the NCI mode (data not shown) [Figure 2].

For bistrifluoromethyl phenyl (BTFMPh) mercury compounds with a trifluoromethyl group detectable in the NCI mode, molecular ions were observed (m/z 406 for BTFMPhMeHg), whereas methyl and ethyl mercury fragments were undetectable [Figure 3]. In contrast, derivatization of inorganic mercury enabled the detection of a specific fragment ion with m/z 630, the molecular weight of $BTFMPh_2Hg$.

No derivative products were observed after the pentafluorophenylation reaction (data not shown). It is considered that the pentafluorophenyl ring is not reactive owing to the electron deficiency by substituted

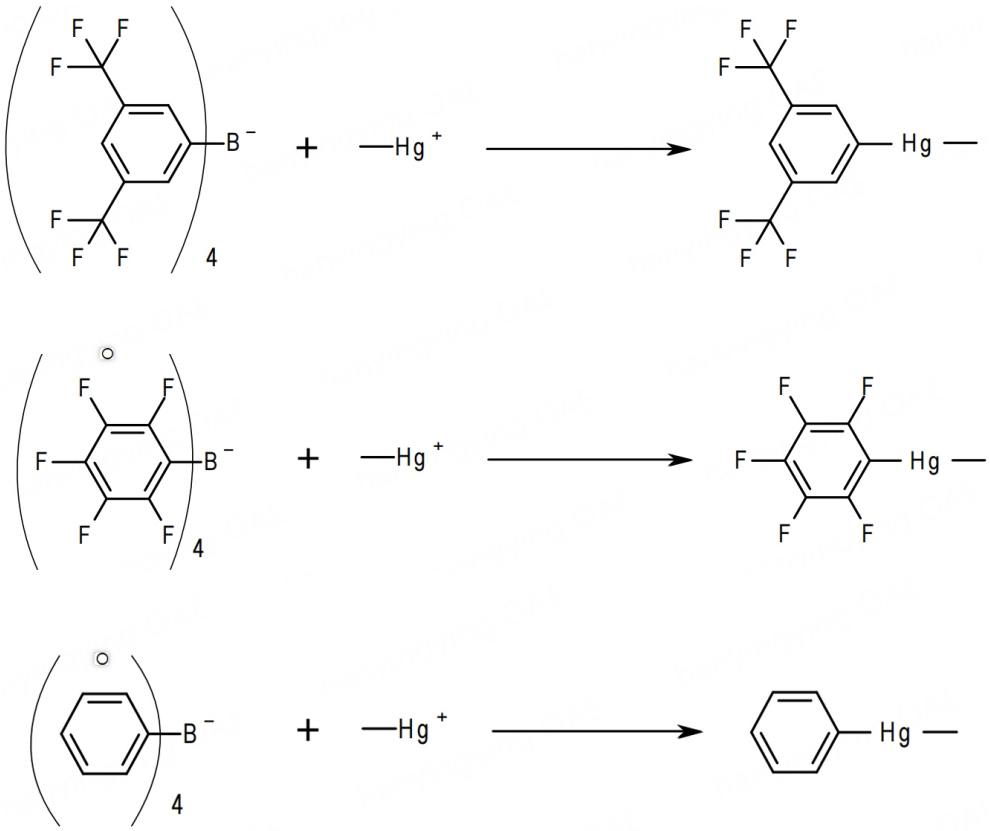

Figure 1. Schemes of the derivatization of mercury compounds with fluorinated tetraaryl borate and tetraphenyl borate salts.

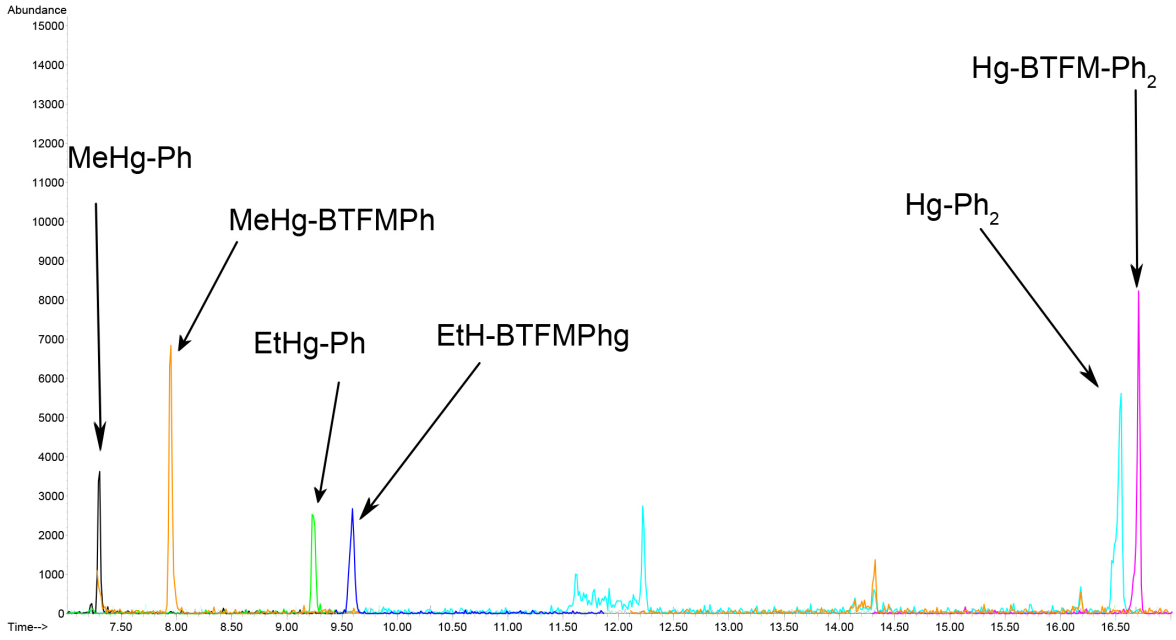

Figure 2. Extracted ion chromatograms of phenylated and bistrifluoromethyl phenylated organic and inorganic mercury. Ph: phenyl group; BTFMPh: 3,5-bis(trifluoromethyl) phenyl group. Mercury compounds in standard solutions were derivatized using sodium tetraphenylborate and sodium tetrakis [3,5-bis(trifluoromethyl) phenyl] borate separately and combined for this analysis. Ions were monitored in the scan mode of electron ionization.

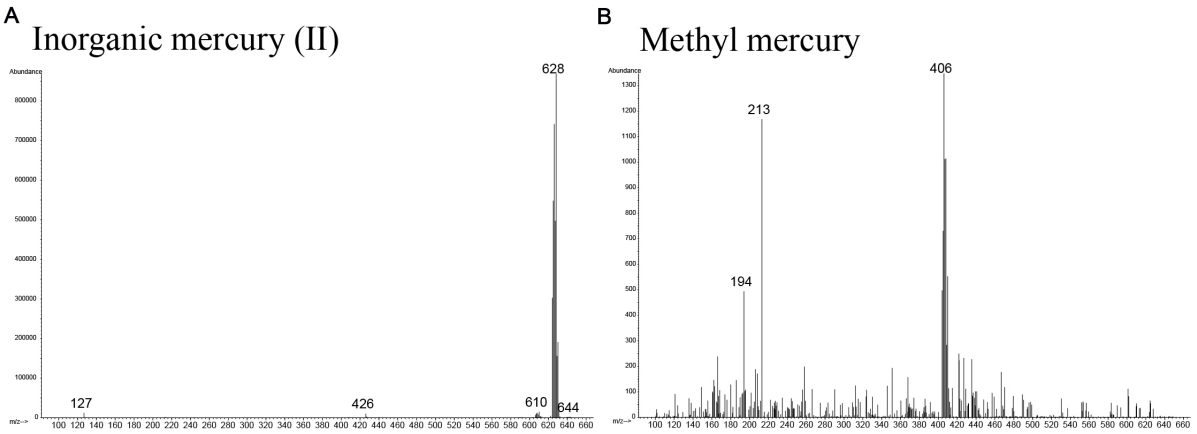

Figure 3. (A): Full-scan mass spectra of BTFMPhMeHg and (B): BTFMPhHg obtained using negative chemical ionization. Both analytes had the same concentration.

fluorine atoms[15].

For mercury chloride, a fragment ion with m/z 237 derived from $[HgCl]^-$ was observed, and the sensitivity was high [Figure 4]. MeHgBr also gave a fragment ion with m/z 281 derived from $[HgBr]^-$. However, MeHgI gave a base peak with m/z 127 from iodine. Among these three halides, MeHgBr is considered applicable to blood samples because it has the highest m/z and is moderately volatile. In contrast, MeHgCl is volatile and thus loss of the analyte during sample preparation can occur.

The analytical procedure is divided into two steps: derivatization of inorganic mercury by $BArF_{24}$ and extraction of organic mercury as a bromide complex.

Stability of derivatized compounds

The stability of derivatized compounds during sample preparation was examined. In the extraction of mercury compounds, cysteine is often used as the reservoir of organic mercury in the water phase owing to strong coordination binding between the sulfhydryl group and mercury[16]. To evaluate the effect of cysteine on the derivatization of inorganic mercury, a standard solution of inorganic mercury (10 μL of 10 μg/mL standard solution) was reacted with $BArF_{24}$ with or without cysteine. The cysteine-containing solution did not contain derivatives (data not shown). In addition, to confirm whether the derivatization was inhibited or whether the reaction with cysteine occurred, a cysteine solution was added to the derivatized standard solution without cysteine. The result showed the absence of derivatized mercury, and cysteine reacted with derivatized mercury because of the nucleophilicity of the sulfhydryl group. Removal of cysteine is required for the analysis of inorganic mercury, although the process has not been established. Analysis of inorganic mercury was not further conducted in this study. Finally, organic mercury was analyzed as a mercury halide complex.

Calibration curves and detection limits

Calibration curves were plotted for MeHgBr and EtHgBr. The linearity (R^2) of the response ratio *vs.* concentration curves was 0.987 for MeHgBr and 0.990 for EtHgBr [Figure 5]. Although a higher response was obtained with a higher concentration, the linearity of the calibration curves between 0.01 ng and 1 ng per sample was 0.978 for MeHgBr and 0.979 for EtHgBr. The instrument detection limits were 0.05 ng and 0.06 ng per sample for MeHgBr and EtHgBr, respectively. The method detection limits in blood matrix were 0.06 ng MeHg per sample and 0.07 ng EtHg per sample. These values correspond to detection limits of

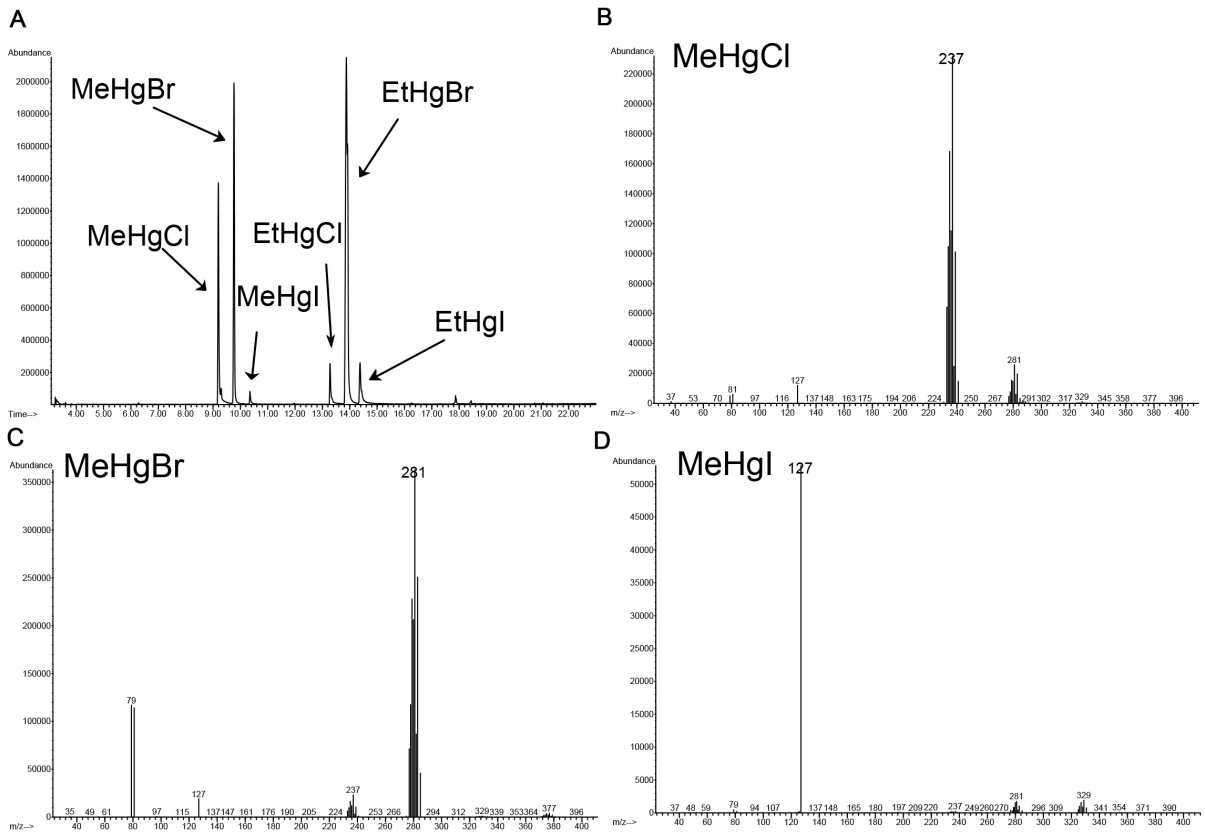

Figure 4. (A): Chromatogram of different halide salts of organic mercury and (B): mass spectra of MeHgCl, (C): MeHgBr, and (D): MeHgI. Organic mercuries in standard solutions were extracted using aqueous solutions containing chloride, bromide, and iodide ions. Spectra were obtained using negative chemical ionization.

0.12 ng/mL (MeHgBr) and 0.14 ng/mL (EtHgBr) per 0.5 mL of blood samples and final extracts.

Sample analysis and validation against GC-ECD analysis

Recovery of organic mercury from whole blood samples with low mercury contamination was evaluated. The blood sample with the lowest mercury concentration (0.66 ng/mL MeHg) was used for this test. Whole blood was spiked at 20 ng/mL with the standard solution (MeHg and EtHg in benzene, 10 μg/mL). The spiked samples were analyzed using the same method for recovery evaluation. The sample signal was subtracted from the spiked sample signal. The recovery of MeHg was 67.1%, while the recovery of EtHg was 49.3% [Table 1]. This difference might be caused by the lipophilicity of the chemicals, and EtHg could be lost during the degreasing process.

The concentrations of MeHg in whole blood samples determined using GC-ECD were used for cross-checking [Figure 6]. The concentrations measured using GC-ECD were in the range of 3.4-32 ng/mL, and the concentrations obtained using GC-NCI-MS were in the range of 1.5-44 ng/mL. The results of the two methods had a correlation coefficient (r) of 0.923. The slope of the best-fit line was 0.80 ± 0.07. Therefore, GC-NCI-MS is applicable for the analysis of MeHg in whole blood samples.

Analysis of a CRM (cod fish tissue)

The concentration of MeHg in a CRM, namely cod fish tissue, was analyzed using GC-NCI-MS. The recovery of MeHg from fish samples was 59% (n = 3). The mean concentration of MeHg determined using

Table 1. Recovery of organic mercury from spiked blood samples

	Whole blood	MeHg-spiked whole blood	Recovery (%)	CV (%)
MeHg	0.66 (0.25)	14.1 (2.92)	67.1	20.8
EtHg	Not detected	9.9 (2.36)	49.3	23.9

Concentration of organic mercury is expressed as the mean (S.D.), n = 7. coefficient of variation (CV) is the ratio of the standard deviation to the mean. MeHg: Methylmercury; EtHg: ethylmercury; CV: coefficient of variation.

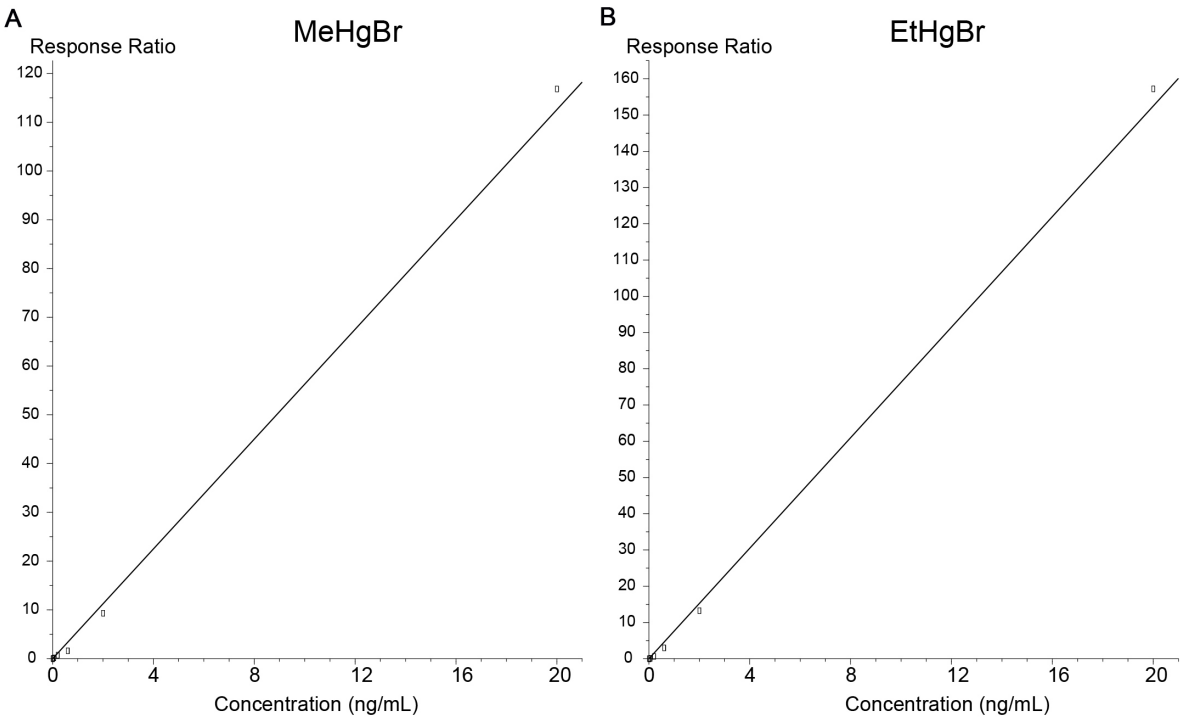

Figure 5. (A): Calibration curves for MeHgBr and (B): EtHgBr. Concentration of analytes and response ratios are based on 0.5 mL samples spiked with the internal standard (10 ng of TCA-d_3). Linear portions were fitted with a weight of the inverse of concentration. Linearity (R^2) was 0.987 for MeHgBr and 0.990 for EtHgBr.

our method was 0.64 μg/g, which agreed with the certified value of 0.58 μg/g [Table 2].

Comparison with existing studies

We aimed to develop a GC-NCI-MS method for the quantitative detection of mercury in blood samples. Zachariadis *et al.* quantified inorganic mercury and MeHg in human urine, saliva, and serum using GC-MS in the EI mode[17]. They applied *in situ* derivatization in aqueous solutions, followed by headspace solid-phase microextraction. This method gave a recovery ranging from 85% to 98% for inorganic mercury and MeHg, and the detection limits for inorganic mercury and MeHg were 10 and 15 ng/L in urine, 54 and 60 ng/L in saliva, and 61 and 81 ng/L in serum, respectively. Watanabe *et al.* evaluated the performance of GC-EI-MS in the detection of MeHg in fish samples[18]. They used sodium tetraphenylborate for derivatization and co-injected polyethylene glycol 200 to suppress the absorption of methyl phenyl mercury within the GC-MS instrument. This method was validated using CRMs and spiked samples, although the detection limit was not determined. Cavalheiro *et al.* compared GC-EI-MS and GC-ICP-MS for monitoring inorganic mercury and MeHg, as well as tin compounds in biological tissues[19]. They used the isotope dilution method and sodium tetrapropylborate for derivatization. The detection limits using GC-EI-MS and GC-ICP-MS were, respectively, 0.079 pg and 0.005 pg for MeHg and 0.041 pg and 0.013 pg for inorganic

Table 2. Concentrations of organic mercury in a certified reference material (NMIJ, CRM 7402-a, cod fish)

	Measured value (μg/g)	CV (%)	Certified value (μg/g)
MeHg	0.64 (0.07)	10.9	0.58 (0.02)
EtHg	Not detected		Not provided

Certified value was provided by the National Metrology Institute of Japan, which was obtained by phenylation- or ethylation-isotope dilution GC-ICP-MS (recovery adjusted in the method). Value determined using GC-NCI-MS was corrected with the recovery of cod fish (59%). Value of organic mercury is expressed as the mean (S.D.), n = 7. MeHg: Methylmercury; EtHg: ethylmercury; CV: coefficient of variation.

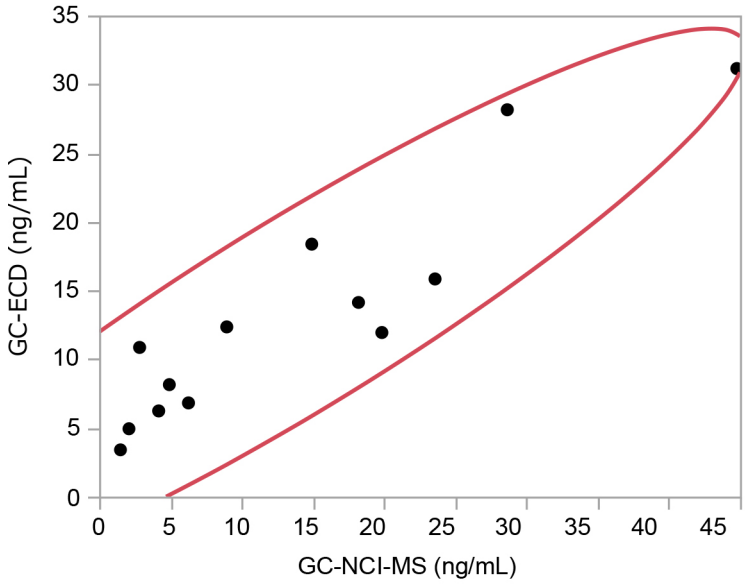

Figure 6. Cross-check of the concentrations of methylmercury in whole blood samples determined using GC-NCI-MS with those determined using the GC-ECD method. n = 13, r = 0.923. Concentrations determined using GC-NCI-MS were corrected for average recovery rates.

mercury. Although GC-ICP-MS performs better than GC-MS, the former requires expensive instrumentation and maintenance. Hippler *et al.* compared a coupled GC-EI-MS/ICP-MS system and a cryo-focusing headspace-GC-MS system for the analysis of MeHg in whole blood samples[20]. The limit of quantification was 0.3 μg/L for GC-EI-MS/ICP-MS and 0.5 μg/L for headspace-GC-MS, and the results obtained using the two methods were in good agreement. In general, GC-MS can offer excellent selectivity and good sensitivity with moderate cost and skill requirements. However, some of the extraction and purification methods provided by the above-mentioned studies are relatively complicated and time-consuming[17,20]. In comparison, our study provides a simpler and lower-cost option for the determination of mercury in biological samples, although further optimization and investigation are needed considering some limitations described below.

Limitations

In this study, we analyzed only blood samples. Analysis of a CRM (cod fish tissue) was conducted, and the recovery from the CRM was slightly lower than that from blood. Although the obtained concentration of MeHg agreed with the certified value, our method needs to be improved to obtain a higher recovery rate, thus enhancing the accuracy and sensitivity. Most of the analytical methods used to determine the levels of mercury species achieve recoveries ranging from 80% to 120%, including the above-mentioned studies[17-18,20]. For other sample matrices, further investigations are needed because the effect of different

sample matrices may affect the analysis result[17].

In addition, optimization of the sample preparation steps is required. This method could not be applied to the analysis of inorganic mercury, and other derivatizing reagents or purification methods to remove cysteine and similar interferents are required. In our extraction procedure, MIBK was used, a Group 2 substance in the Ordinance on Prevention of Hazards Due to Specified Chemical Substances. Safe organic solvents will be examined instead of MIBK in future studies.

CONCLUSION

We investigated the use of a derivatization method and GC-NCI-MS for the quantitative detection of organic and inorganic mercury in blood samples. GC-NCI-MS equipped with a capillary column could detect MeHg and EtHg bromide complexes in blood samples. The detection limits were 0.12 ng/mL and 0.14 ng/mL for MeHgBr and EtHgBr, respectively. The recovery of MeHg and EtHg from whole blood samples was 67.1% and 49.3% (CV%: 20.8% and 23.9%), respectively. The values obtained using our developed method agreed with the values obtained using GC-ECD and the certified value of the CRM.

DECLARATIONS

Acknowledgments

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Authors' Contributions

Made substantial contributions to the conception and design of the study: Li M, Harada KH
Performed data analysis and interpretation: Lyu Z, Soleman SR, Li M
Performed data acquisition: Lyu Z, Soleman SR, Li M
Provided administrative, technical, and material support: Harada KH

Availability of Data and Materials

All the data were included in this paper. No additional data are available.

Financial Support and Sponsorship

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Conflicts of Interest

All authors declared that there are no conflicts of interest.

Ethical Approval and Consent to Participate

Written informed consent was obtained from individuals before the blood sampling. This analysis is approved by the Kyoto University Ethics Committee (approved no. R1478).

Consent for Publication

Not applicable.

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