Methylmercury Measurement in Whole Blood by Isotope-Dilution GC-ICPMS with 2 Sample Preparation Methods

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Background: Despite its known toxicity, methylmercury is rarely measured directly in clinical studies; instead, conclusions are based on total mercury measurements. We have developed isotope–dilution-based methods for methylmercury-specific analysis of whole blood by coupled gas chromatography–inductively coupled plasma mass spectrometry (GC-ICPMS).

Methods: We analyzed animal and human blood samples after alkaline digestion or extraction of methylmercury into dichloromethane and back-extraction into water. Methylmercury was converted to the volatile ethyl derivative, purged, and trapped on a solid-phase collection medium, and then introduced into the GC-ICPMS system.

Results: Limits of quantification were 0.4 and 0.03 μg/L at a signal-to-noise ratio of 10 with the alkaline digestion and extraction methods, respectively. Extraction met our selected acceptable total error criterion, with an SD of 0.58 μg/L at the critical maternal blood concentration of 5.8 μg/L. For 37 blood samples, the mean (SD) proportion of total mercury present as methylmercury was 60 (27)%, range 6%–100%.

Conclusions: The combination of extraction and isotope-dilution GC-ICPMS meets the requirements for use as a reference method for measuring methylmercury in whole blood.

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In humans, fish consumption is the major source of methylmercury, a naturally occurring neurotoxin (1–3). Methylmercury’s ability to cross biological barriers is of particular concern because it can pass from the placenta to the fetus, potentially impairing neurological development (1). Numerous investigations conducted to assess the health risks of prenatal exposure to methylmercury [collected by Rice (4)] have led to the establishment of a critical maternal blood concentration of 5.8 μg/L (29 nmol/L) (2, 3). Further effects of chronic, low-level exposure to methylmercury, such as increased incidence of heart disease in men (5, 6) and delayed neurotoxicity (7), are also being recognized. Thus, there is a growing need for a method to measure methylmercury in clinical specimens.

Most clinical studies cite whole blood and hair as optimal biomarkers for mercury, and although the total mercury concentration in hair is often assumed to average 250-fold higher than that in blood, a value employed in pharmacokinetic models, considerable variation exists (1, 4, 8, 9). Although methylmercury is the predominant form in blood (1, 3), inorganic mercury is also present, and its concentration may be increased by occupational exposure (1). For these reasons, the specific measurement of methylmercury in blood must be the preferred biomarker.

Although several methods have been developed for measuring methylmercury in blood (10–13), these methods are all fairly complicated, and the analytical techniques most commonly applied in clinical laboratories measure total mercury concentrations. A simpler method, although not specific for methylmercury, is to differentiate inorganic from total mercury by selective reduction with SnCl₂ or SnCl₂ + CdCl₂, respectively (1, 14). The elemental mercury vapor generated can then be detected by a variety of analytical techniques, with atomic fluorescence spectrometry being popular because of the relatively inexpensive instrumentation required, coupled with very favorable limits of quantification (3). The use of
toxic CdCl₂ creates hazardous waste, however, and as noted above, methylmercury is not measured directly.

We modified a previous method based on digestion of blood in methanolic KOH (13) to incorporate isotope dilution (15), resulting in a more robust analytical system employing gas chromatography–inductively coupled plasma mass spectrometry (GC-ICPMS) for separation and detection of methylmercury (16). Like the earlier method, the detection capabilities of the new method were limited by matrix effects, and we also evaluated an alternative sample preparation protocol, previously applied to the measurement of methylmercury in various sample types (11, 16, 17). By extraction of methylmercury into dichloromethane, and back extraction into water, this preparation method eliminates matrix effects, but sample preparation is more laborious. We compared methods with respect to results obtained for clinical samples and commercially available blood materials, as well as limits of quantification.

Materials and Methods

INSTRUMENTATION

Measurement of mercury species. Quartz tubes, 25 cm × 2 mm i.d., were packed with a 30-mm plug of Tenax TA (mesh 60–80, Supelco) held in place with quartz wool. A 10-cm length of uncoated fused-silica capillary column (0.32 mm i.d., Supelco) was attached to one end of the quartz tube to serve as a needle for injecting mercury species, trapped on the Tenax, into the GC-ICPMS instrument. The other end of the quartz tube was fitted with flexible tubing to allow connection to an argon gas supply system plumbed via the GC with a head pressure of 100 kPa. To thermally desorb mercury species collected in the Tenax tube, a laboratory–designed oven was used, mounted above the injection port of the gas chromograph.

The GC-ICPMS system consisted of a Fisons Instruments (now Thermo Electron) 8000 Series gas chromatograph equipped with a 15-m capillary column (0.53 mm i.d., 1.5 μm BP-1, Supelco) and coupled to an X Series ICPMS system (Thermo Elemental) through a heated interface (200 °C), constructed in the laboratory according to a previous description (16). (Thermal desorption and coupled GC-ICPMS systems are commercially available.)

The injector of the GC was maintained at 120 °C, with column inlet pressure of 28 kPa argon in the splitless mode for 175 s. During thermal desorption of mercury species from the Tenax tube, the oven temperature was held at 34 °C for 2 min and then ramped to 90 °C at the maximum rate of 49.9 °C/min, with 0 min hold time. The ICPMS instrument was operated at a forward power of 1200 W, with other parameters being optimized during continuous infusion of elemental mercury from a vapor generation device (PS Analytical). Data acquisition commenced 120 s after the start of the thermal desorption step in the transient time-resolved analysis mode, and continued for 200 s while monitoring the 198Hg, 200Hg, and 202Hg isotopes with dwell times of 100 ms on each. The instrumental software was used to calculate baseline-corrected peak areas.

Measurements of total elemental concentrations. Digestion of 1 mL blood mixed with 1 mL of 14 mol/L nitric acid was accomplished with a microwave oven (MD-2000, CEM Corporation) equipped with 12 low-volume PFA lined vessels with safety rupture membranes for 1 h at 600 W power. The digest was diluted to 20 mL with deionized water (from a MilliQ system). Two or more blank digests that used 1 mL of water instead of blood were also prepared with each batch of blood samples. The resulting solutions were enriched to 25 μg/L with internal standard solution containing In and Tl. Total elemental concentrations were determined by ICPMS with a high-resolution instrument (Element, Thermo Finnigan), as detailed elsewhere (18, 19). Quality control was achieved by routine analysis of certified reference materials and regular participation in international quality assessment programs since 1995 (20).

BLOOD SAMPLES

Several batches of lyophilized blood (Seronorm Trace Elements Whole Blood) were obtained from Sero AS, Billingstad, Norway; A-13 (Freeze-Dried Animal Blood) was purchased from the International Atomic Energy Agency. For the preparation of blood pools for assessing within-run and between-day imprecision (see the Supplemental Data file that accompanies the online version of this article at http://www.clinchem.org/content/vol53/issue1), we acquired bovine blood from a local retailer.

Human blood samples used in the method comparison study were residual aliquots of pools prepared in-house for other purposes, material remaining from various interlaboratory trials, or samples submitted for mercury measurement such as those from Seronorm.

REAGENTS AND PROCEDURES

All sample manipulations were performed in laminar flow fume cupboards in a laboratory supplied with HEPA-filtered air to minimize contamination risks.

Sodium tetraethyl borate (5 g) was purchased from Galab Technologies and dissolved in 20 mL tetrahydrofuran (VWR International) that was injected directly into the septum-equipped container in 5 mL portions while cooling. Equal volumes of gas were removed from the container after each addition. We then injected 0.4 mL of argon gas aliquots with a gas-tight syringe (Hamilton Co.), removed an equal volume of reagent, and transferred the reagent to a 2-mL glass GC vial containing 1.5 mL deionized water. All sodium tetraethylborate solutions were stored frozen at −20 °C until further use.

An isotopically enriched (to 96.36% in the CH₃¹⁹⁸HgCl isotopomer) methylmercury chloride in methanol solution of nominal concentration 5 mg/L (as Hg) was obtained from the National Research Council Canada (21).
This stock was diluted in deionized water to provide an enriching solution of 10 μg/L when required, the exact concentration being determined by reverse isotope dilution (22). Intermittent checks of 10-fold diluted enriched solutions indicated that <5% degradation occurs during a period of 3 months of storage in 2 mL glass GC vials at 5 °C. We used a certified aqueous solution of methylmercury chloride (1.00 g/L as Hg; Alfa Aesar) traceable to NIST SRM 3133, as the calibrant. We performed calculations of methylmercury concentrations with the isotope dilution equation in accordance with the mathematical approach developed by Yang and Sturgeon [22] and see the online Supplemental Data.

We purchased an independent standard solution (10 mg methylmercury chloride/L in acetonitrile) from LGC Promochem as a control material.

Alkaline digestion method. A methanolic KOH solution was prepared by dissolving 25 g potassium hydroxide (EKA Chemicals) in 100 mL methanol (Merck), in 25 mL portions, while cooling. After an overnight incubation, the solution was filtered through 0.45 μm membrane filters (Sarstedt). Blood samples (0.5 mL) were transferred, using calibrated pipettes, into 50 mL polypropylene centrifuge tubes (Sarstedt) to which 500 μL of enriched solution was added. After addition of 5 mL methanolic KOH solution, the samples were digested in an ultrasonic bath (T-660/H, Elma) for 4 h, 1 mL of 1.0 mol/L copper sulfate solution was added, and 200 μL aliquots of blood into 50 mL centrifuge tubes, and then centrifuged at 2000 g. After centrifugation at 2000 g, the samples were digested in an ultrasonic bath (T-660/H, Elma) for 4 h (13, 22). Blanks and calibrators were treated in a similar manner.

Extraction method. We pipetted 1 mL, or up to 2.5 mL, aliquots of blood into 50 mL centrifuge tubes, and then added 200 μL of enriched solution. After equilibration for 4 h, 1 mL of 1.0 mol/L copper sulfate solution was added, followed by 5.0 mL of 1.4 mol/L potassium bromide (Sigma-Aldrich) in 0.9 mol/L sulfuric acid (Merck) and 10 mL dichloromethane (16). The tubes were rotated on an overhead mixer (Heidolph) for 1 h to extract methylmercury. After centrifugation at 2000g for 15 min (Megafuge 1.0, Heraeus), the dichloromethane was transferred to a fresh 50 mL polypropylene tube with a glass Pasteur pipette.

Deionized water (10 mL) was added to the dichloromethane phase, and the tube was placed in a beaker of hot water. Argon gas was then bubbled through the denser organic phase for ~10 min, until the dichloromethane was completely evaporated. Blanks and calibrators were treated in a similar manner.

Ethylation of mercury species. We added 100 mL of deionized water and 1 mL of 1 mol/L pH 5 citrate buffer (23) to a 125 mL silanized glass Dreschel bottle (Sigma-Aldrich), followed by 1 mL alkaline digested material (plus ~200 μL distilled nitric acid to obtain an optimum pH of 5) or 3 mL of the aqueous extract. Then 25 mL (extraction method) or 100 μL (KOH digestion method) of sodium tetraethyl borate solution was added, the Dreschel bottle was closed, and the ethylated mercury species formed by the reaction were purged from solution by an argon flow of 130 mL/min for 10 min. The ethylated mercury species were collected on the Tenax tube after passing through a Nafion drying tube (PS Analytical).

Performance of the methods
Linearity for samples, prepared by the KOH digestion and extraction methods, was verified up to 100 μg/L and 15 μg/L, respectively, in calibration experiments. Within- and between-run imprecision data for both methods were estimated from duplicate analyses of a synthetic control material diluted to 5.8 μg/L (equivalent to the medical decision level) on at least 20 days. We also assessed the performance of each method by adopting the same proficiency-testing requirement as for blood lead, i.e., achieving the target value to within 10% (24). In terms of defining a performance standard (25), this translates into a maximum acceptable total error of 0.58 μg/L at the medical decision level of 5.8 μg/L.

Results and Discussion
For the KOH digestion method, the within- and between-run imprecision levels were 3.5% and 3.8%, respectively; the corresponding Figs. for extraction were 1.9% and 3.8%. Additional data from imprecision studies and evaluation of linear dynamic ranges are provided online in the Supplemental Data.

The extraction procedure fulfilled all requirements, although the total analytical error for the KOH digestion method, at 0.70 μg/L, exceeded the acceptable error. Subsequent modification of the method allowed the problem identified during the trials to be rectified, as described in the online Supplemental Data. A comparison of the KOH digestion and extraction methods, based on regression analysis (26) and the Bland-Altman difference plot (27), is also provided in the online Supplemental Data.

A comparison of results for methylmercury obtained using a number of commercially available materials is shown in Table 1. Although not certified for their methylmercury concentrations, several of these materials remain available and thus may be of value for future comparisons. Interestingly, the results obtained by both isotope-dilution based GC-ICPMS methods are within the range obtained with selective reduction, assuming that the difference is entirely attributable to methylmercury (3, 28). Because the concentration of methylmercury in the material derived from animal blood was expected to be very low, aliquots equivalent to ~2.5 mL of reconstituted sample were processed by the extraction method. The resulting chromatograms, exemplified in Fig. 1A, demonstrated that the methylmercury peak was readily distinguishable from the blank.

Limits of quantification were first approximated as the methylmercury concentrations yielding signal-to-noise ratios of 10. This resulted in estimates of 0.4 and 0.03 μg/L
using KOH digestion and extraction methods, respectively, with the latter value comparing favorably to all previously described techniques for the determination of methylmercury in blood (3, 10–14). To confirm the limits of quantification, IAEA A-13 was used as matrix because this contained the least methylmercury of the available blood materials. Before KOH digestion, the lyophilized animal blood material was enriched with natural abundance methylmercury to yield an expected concentration of 0.431 μg/L (from the added plus incipient concentrations given in Table 1). For the extraction method, aliquots of IAEA A-13, corresponding to the normal sample intake of 1.0 mL blood, were processed directly without standard addition. The results included in Table 1 confirm the aforementioned limits of quantification, based on an imprecision of <20% and a trueness to target within 20% for both methods.

As discussed by Liang et al. (13), suppression of the ethylation reaction is the limiting factor in the analysis of KOH digests of blood. The improved limit of quantification after extraction thus results from the use of a greater proportion of the initial sample volume for ethylation (30% is used routinely, although >90% is possible). Semi-quantitative analyses of aqueous back-extracts, performed by high resolution ICPMS (18, 19), proved the efficacy of the clean-up procedure, because only 6 elements of >60 monitored were consistently found at concentrations corresponding to >1 mg/L in blood. Of these, Br, K, S, and Cu (at ~60, ~10, ~6, and ~3 mg/L, respectively) likely originate from reagents added to the blood, and Cl (at the percentage level) derives from residual dichloromethane in the aqueous phase. The latter hypothesis is supported by the fact that increased concentrations of chloride salts would depress the ethylation reaction (17), an effect not seen in practice, and by the lack of sufficient cationic species in the extract to balance the Cl concentration, if assumed to be present in the form of chlorides. Therefore, only P (at ~2 mg/L) is exclusively derived from blood, albeit with exceedingly low recovery. The purity of the blood extract is thus conducive to high ethylation efficiency.

Representative chromatograms obtained for blood samples containing methylmercury concentrations 3 to 6 times the limits of quantification, and demonstrating the baseline separation of the analyte from inorganic mercury, are shown in Fig. 1. The formation of volatile ethylated mercury species, in combination with their gas chromatographic separation before mass spectrometric detection, effectively eliminates all potentially interfering species.

The value of methylmercury-specific data compared with total mercury data is demonstrated in Fig. 2, which displays results for a number of samples, including both kinds of information. Note that only one data point in Fig. 2 lies above the line of identity, although the methylmercury and total mercury concentrations are not significantly different, providing an additional internal quality control variable for the methods in use in the laboratory. The mean (SD) proportion of total mercury present in the form of methylmercury for the sample shown in Fig. 2 is 60 (27)% (1 SD uncertainty, n = 37), with a range of 6%–100% and a median of 64%. Together with the observation of a mean of 49% in a contemporary study (29), these results strongly indicate that total mercury in blood is a very poor surrogate for the methylmercury concentration.

The 2 highest total mercury concentrations in Fig. 2 were flagged by the laboratory information management system as exceeding the upper limit of the reference interval (18, 30). These blood samples originated from persons suspected of being occupationally exposed to inorganic mercury, as often occurs in the working environment (1). Such investigations rely on measurements of total mercury although, as clearly evident from the meth-

### Table 1. Mercury in commercially available blood materials.

<table>
<thead>
<tr>
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<td>1.39 (0.08)</td>
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<td>1.2 (3); 1.9 (28)</td>
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Recovery, %

1. IAEA A-13 + 0.4 μg/L: 0.476 (0.050)f

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- a: Seronorm lyophilized human whole blood samples. Uncertainties in methylmercury concentrations (given in parentheses) are expressed at the 2 SD level for duplicate analyses of pairs of sample bottles.
- b: Calculated as the difference between determined values for total and inorganic mercury.
- c: Data from the certificate of analysis.
- d: Measured (1 SD) value for triplicate preparations of 250 mg lyophilized animal blood was 0.33 (0.04) μg/kg, expressed as reconstituted material by dividing by 10.5 L/kg (19).
- e: Measured (1 SD) value for 6 replicates using 100 mg lyophilized animal blood reconstituted in 1.000 mL water.
- f: Measured (1 SD) value for 6 replicates using 50 mg lyophilized animal blood spiked with 0.20 ng (as Hg) methylmercury and reconstituted in 0.500 mL water.
ylmercury data, one of the sample donors, represented by the data point in the upper right quadrant in Fig. 2, is unlikely to have been recently occupationally exposed. Instead, this individual is an avid seafood consumer. Excluding the latter data point, the mean (SD) methylmercury concentration is 1.32 (1.10) μg/L, with a range of 0.15–4.48 μg/L and a median of 1.14 μg/L. These values are comparable to the mean (SD) concentration of 1.21 (1.00) μg/L and range of 0.20–4.98 μg/L reported by Kobal et al. (29) for a sample set of 58 controls.

Two samples had mean (SD) total mercury concentrations of 6.1 (0.3) μg/L and 6.8 (0.3) μg/L above the critical maternal blood concentration. The corresponding methylmercury concentrations were 4.2 (0.2) μg/L and 4.0 (0.2) μg/L, both significantly below the critical concentration. Thus the 2 types of mercury data could lead to different medical decisions.

Our results suggest that both of the isotope-dilution GC-ICPMS–based methods described here can measure methylmercury at the critical maternal blood concentration of 5.8 μg/L, with total error <10% (25). During 18 months of testing, the presence of methylmercury has not been missed in any sample. Extraction of 1 mL blood yields a limit of quantification of 0.03 μg/L. Further improvements in the limit of quantification can be realized by processing larger sample volumes (up to 2.5 mL has been tested) or by subjecting the entire 10-mL aqueous extract to ethylation, rather than 3 mL, as routinely used. The latter approach could also be used to partially compensate for losses in detection capability should limited sample volumes be available, as in the case of cord blood.

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References