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Determination of methylmercury in human blood using capillary gas chromatography and selected-ion monitoring

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ABSTRACT

A capillary gas chromatographic method, using selected-ion monitoring in the electron-impact mode, was developed for the analysis of methylmercury (MeHg) in human blood. The samples, spiked with the internal standard propylmercury bromide (PropHgBr), were, after addition of sodium bromide and cupric sulfate, extracted with toluene. The organic phase was extracted with an ethanol–water solution of sodium thiosulfate. After addition of sodium bromide solution, the ethanol water phase was extracted with toluene. Methylated derivatives ($MeHgCH_2Br$ and $PropHgCH_2Br$) were formed by the addition of a diethyl ether solution of diazomethane. The chromatographic properties of the derivatives were much better than those of the non-methylated compounds. The *m/z* 215 fragment of $MeHgCH_2Br$ and the molecular ion *m/z* 338 of $PropHgCH_2Br$ were monitored. The calibration graphs, with a linear correlation coefficient of 0.992 ($n = 12$) in the 1–5 μg/l concentration range, passed through the origin. The detection limit for MeHg in human blood was *ca.* 0.5 μg/l. Analysis of spiked blood samples at concentrations of about 2 and 10 μg/l gave a relative standard deviation of 4.2 and 5.5%, respectively ($n = 10$).

INTRODUCTION

Exposure to mercury (Hg) is prevalent, both in the external and in the work environment [1]. The species of greatest interest is inorganic mercury, exposure to which occurs from amalgam fillings and in several occupational settings, and methylmercury (MeHg), which is the dominating form present in fish. These two chemical forms have a quite different toxicology. There is thus a great need for methods for speciation of mercury in biological materials. Such methods should be sensitive enough to cover the low levels found in samples from "normal" subjects; in blood this means levels down to about 1 μg/l, or even less [2].

Gas chromatography (GC) with packed or capillary columns is the most used technique for the determination of volatile organic mercury compounds. The methods are generally based on the formation of methylmercury halogenide (MeHgX) in acidic aqueous bromide or chloride solution, and extraction into benzene or toluene. For sample clean-up, MeHg is extracted into a cysteine [3] or thiosulfate [4,5] aqueous phase. After separation of the phases, halogenide is added again and MeHgX is back-extracted into the organic solvent. Ethylmercury has been used as internal standard added to the biological sample before the work-up procedure [5].

Adsorption and decomposition of MeHgX in the chromatographic system has been demonstrated to present a serious problem. It was observed in a gas chromatographic–mass spectrometric (GC–MS) study with packed columns [6] that, after injection of methylmercury chloride (MeHgCl), substitution of the halogenides occurred with the formation of methylmercury iodide (MeHgI). Similarly, when MeHgI was injected, the formation of MeHgCl was found. The addition of mercuric chloride to the samples or repeated injections of microgram amounts of MeHg improved the chromatography [7]. Glass capillary columns coated with OV-275 have been

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found to be efficient and no adsorptive behavior was observed [8].

Detection has mainly been performed using electron-capture detection (ECD) [7,8], with detection limits in the range of 0.2 pg of MeHgCl per injection. However, this type of detector is very sensitive to interferences from solvent impurities. A decrease in peak height due to contamination of the ECD foil has been reported [9]. Therefore, standards have to be injected every few samples.

Detection with cold vapor atomic fluorescence [10], microwave-induced helium plasma emission [11] and cold vapor atomic absorption spectrometry (CV-AAS) [12,13] has also been used. MS has been used to identify MeHg in aquatic organisms [14].

Aqueous phase ethylation of water samples, analyzed with cryogenic gas chromatography and cold vapor atomic fluorescence detection, has been shown to give detection limits of about 3 pg/l mercury in water [15].

Liquid chromatography (LC) with UV detection for the determination of organic mercury compounds such as dithizone [16] or alkyldithiocarbamate [17,18] complexes has been demonstrated to give a detection limit below 2 μg/l MeHg in urine [16].

In this paper we present a modification of the Westöö [3] method, further developed by Goolvard and Smith [5]. Our modification includes a derivatization procedure with diazomethane, with the formation of methylmercurymethylene bromide ($MeHgCH_2Br$), and determination by gas chromatography–selected-ion monitoring (GC SIM). Propylmercury bromide (PropHgBr) was used as internal standard.

EXPERIMENTAL

Apparatus

A quadrupole mass spectrometer (GCMS-QP1000 EI/CI, Shimadzu, Kyoto, Japan) connected to a gas chromatograph (Shimadzu GC-9A) equipped with a fused-silica capillary column, an autosampler (Shimadzu AOC-9) and a split- splitless injection system (Shimadzu, SPL-G9) was used for identification and quantification. The column (30 m × 0.24 mm I.D.) was obtained from J & W Scientific (Folsom, CA, USA) and contained a bonded stationary phase, DB-5 (0.25 μm).

The GC injector temperature was 160°C. The temperature program for the column oven was: 85°C isothermal for 2 min, increasing by 20°C/min to 200°C, isothermal for 1 min. The split exit valve was kept closed for 1 min after the injection. The carrier gas was helium with an inlet pressure of 1.0 kg/cm^2.

The column outlet was mounted directly into the ion source. The temperature of the GC–MS interface and the ion source was 200°C. The instrument was used in the electron-impact (EI) mode (70 eV). The ion source filament was turned on after 4 min and off after 7.2 min. Five measurements were made every 2 s (rate 2).

Chemicals

Chemicals used were methylmercury chloride, sodiumthiosulfate, diethyl ether, sulfuric acid and potassium hydroxide from Merck (Darmstadt, Germany), N-methyl-N′-nitro-N-nitrosoguanidine from Fluka (Buchs, Switzerland), toluene from Lab-Scan (Dublin, Ireland), ethanol from Kemetyl (Stockholm, Sweden) and copper sulfate and sodium bromide from Janssen Chimica (Geel, Belgium). PropHgBr can be obtained from Syntelec (Lund, Sweden).

Derivatization reagent solution

An Erlenmeyer flask containing a mixture of 15 ml of diethyl ether and 4.5 ml of a 45% aqueous potassium hydroxide solution was stirred and kept at *ca.* 5°C. N-Methyl-N′-nitro-N-nitrosoguanidine (2.1 g) was added in small portions. The formed diazomethane (*ca.* 0.42 g) was dissolved in the organic phase, which turned yellow. The diethyl ether solution of diazomethane was separated and transferred to a 20-ml test tube. The reagent solution was dried with granulated potassium hydroxide overnight.

Standard solutions

An accurately weighed amount (*ca.* 35 mg) of MeHgCl was dissolved in 250 ml of water. The internal standard, PropHgBr (*ca.* 20 mg), was dissolved in 100 ml of acetonitrile. The solutions were further diluted with water.

Sampling

Venous blood was sampled in heparinized test tubes (Venoject). For the determination of MeHg in blood cells, the sample was centrifuged and separated. The samples were stored at −20°C until analysis.

Work-up procedure

A 2-ml sample of whole blood or blood cells and 100 μl of the internal standard (250 ng/ml PropHgBr) were transfered to a 100-ml glass tube with screw cap and PTFE gasket. A 15-ml aliquot of 0.1 *M* aqueous copper sulfate solution, 20 ml of a sodium bromide solution [442 g of sodium bromide and 315 ml of sulfuric acid, 50% (v/v), diluted with 1 l of water] and 30 ml of toluene were added. The mixture was shaken for 10 min and centrifuged (1200 *g*) for 10 min. The organic phase was transferred to a 50-ml centrifuge tube, and MeHg was extracted (shaking 10 min) into 0.5 ml of thiosulfate solution [124 mg of sodiumthiosulfate per liter of 50:50 (v/v) ethanol–water]. The mixture was centrifuged (2000 *g*) for 5 min and the thiosulfate solution was transferred to a 4-ml test tube. The extraction was repeated once. To the combined extracts, 1 ml of the sodium bromide solution and 0.5 ml of toluene were added. The mixture was shaken for 10 min and the organic phase was transferred to a 2-ml test tube. $MeHgCH_2Br$ and $PropHgCH_2Br$ were formed by the addition of 100 μl of the derivatization reagent solution. Each sample was prepared in duplicate.

Mass spectrometric determination

The fragment ion ($MeHg^+$) *m/z* 215 of $MeHgCH_2Br$ and the molecular ion (M^+) *m/z* 338 of the internal standard $PropHgCH_2Br$ were monitored by SIM. The average of the ratios between these measurements from triple injections was used for quantification.

Comparison with levels of total mercury and inorganic/ionic mercury levels

Total mercury was determined in wet digested samples by an automated CV-AAS method according to Einarsson *et al.* [19]. Blood cells and whole blood (0.5 g) were digested with concentrated nitric (0.5 ml) and perchloric (2.5 ml) acids at 65°C overnight.

Inorganic/ionic mercury was determined by CV-AAS according to Velghe *et al.* [20]. The method was modified as follows: 3 ml of 16 *M* sulfuric acid were added to the sample (0.5 g of whole blood or 0.3 g of blood cells diluted with 0.3 ml of water) during continuous shaking and air cooling of the reaction vessel (a midget impinger bubbler). After approximately 10 min, 2.0 ml of water were added. The mercury fraction reducible by tin(II) was determined after addition of 0.20 ml of hydroxylammonium hydrochloride solution (25%, w/v) and 0.25 ml of stannous chloride solution [10% (w/v) in 1.8 *M* sulfuric acid]. Octanol (one drop) was added to avoid excessive foaming. Before aeration of mercury(0) into the gas cell, the reaction vessel was shaken vigorously for 20 s on a whirl mixer to equilibrate mercury(0) between the sample solution and the gas phase of the reaction vessel, thus increasing the sensitivity (peak height).

RESULTS AND DISCUSSION

Chromatography and speciation of methylmercury

Standards. Standards of MeHgCl and PropHgBr were identified by GC–MS and the purity was checked by GC–flame ionization detection (GC-FID) and AAS. The purity was found to be better than 96%. PropHgBr was choosen as internal standard because of the similarity in chemical structure and MS fragmentation (see Fig. 1). PropHg compounds are not expected to occur in natural biological samples.

Work-up procedure. The ratio between MeHg and PropHg was constant during repeated analysis of a whole-blood sample spiked with MeHg (μg/l), indicating a very similar recovery of both compounds. The addition of copper(II) sulfate is reported to release MeHg from strong bonds with sulfur in biological samples [4]. The clean-up extraction with thiosulfate is to exclude blood lipids from the final extract. The derivatization with diazomethane was studied by comparing chromatograms obtained for a toluene solution containing a high concentration of MeHgBr (*ca.* 50 μg/ml) with those of the same solution (1 ml) after the addition of 200 μl of the derivatization reagent solution. In the derivatized sample the MeHgBr peak disappeared and a $MeHgCH_2Br$ peak appeared. No other peaks arose. When a

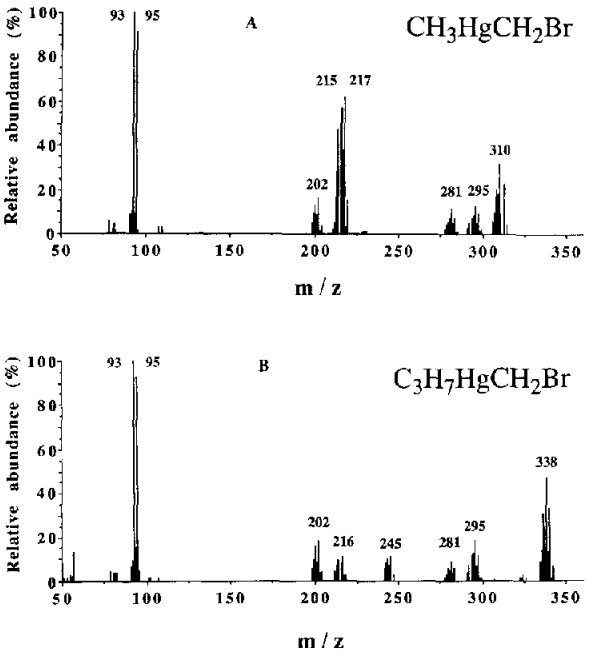

Fig. 1. Mass spectra obtained with electron impact at an ionization potential of 70 eV. (A) Methylmercury derivative; (B) propylmercury derivative.

dilute toluene solution of MeHgBr (10 μg/l) was derivatized and analyzed by GC–SIM, the same result was found. When analyzing aqueous solutions and whole blood and blood cells, respectively, spiked with MeHg and PropHg, no noticeable differences in the results were found.

When blood samples containing *ca.* 2 μg/ml MeHg were spiked with mercuric chloride to a concentration of 25 μg/l, no increase in the $MeHgCH_2Br/PropHgCH_2Br$ ratio was found. When spiked with 500 ng of PropHgBr (twenty times the amount used in the procedure), the change in the ratio indicated a MeHg contamination corresponding to less than 1 μg per liter of blood. This indicates that the normal amount of the internal standard has a completely negligible influence on the MeHg level.

Owing to the tedious work-up procedure, only about ten samples can be analyzed per day.

Chromatography. MeHgBr, PropHgBr and their corresponding methylated derivatives were studied using GC–SIM. MeHgBr showed a tailing peak with considerable adsorptive behavior in the chromatographic system at concentrations in the low μg/l level. Further, the peak eluted on the tail of the solvent peak. No column adsorption was found for $MeHgCH_2Br$, which eluted with a symmetrical peak, well separated from the solvent peak. PropHgBr and $PropHgCH_2Br$ gave symmetrical peaks, well separated from the solvent peak. No column adsorption was observed for these compounds.

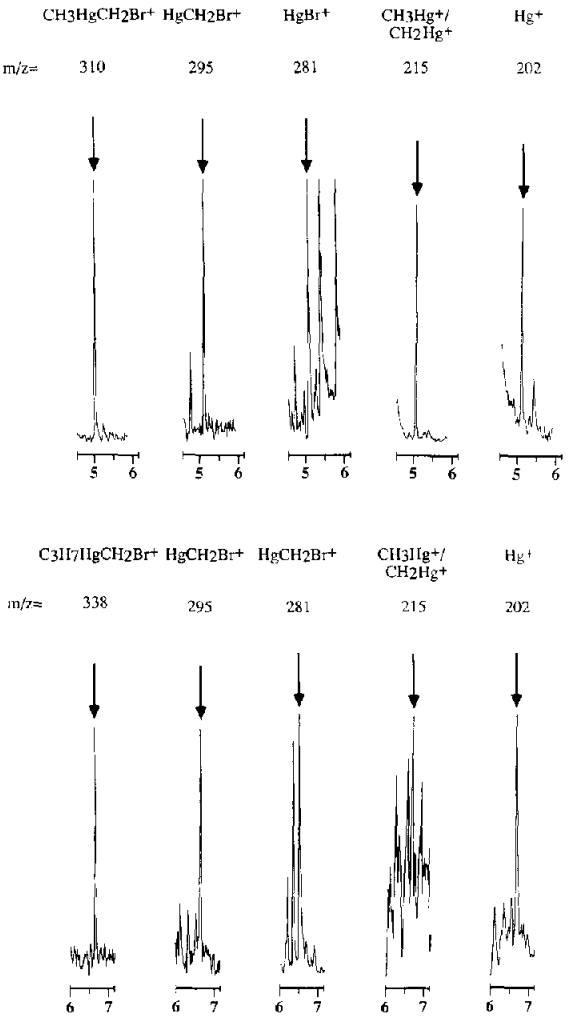

Fig. 2. Multiple-ion monitoring of methylene bromide derivatives of methylmercury (top line) and the internal standard propylmercury (bottom line) from blood containing *ca.* 15 μg/l methylmercury and 12.5 μg/l propylmercury using the electron-impact ionization mode (70 eV). The ion source was at 200°C. Column: J & W fused-silica coated with DB-5 bonded stationary phase (30 m × 0.243 mm I.D.), 0.25 μm film thickness. Inlet pressure of the carrier gas (helium): 1.0 kg/cm^2. Splitless injection (1 min) of 5 μl of toluene. Temperature programming: 85°C isothermal for 2 min, increasing by 20°C/min to 200°C, isothermal for 1 min.

Mass spectrometry. The structures of the diazomethane derivatives of MeHgBr and PropHgBr were confirmed by GC–MS in the EI mode, which showed that the bromide was exchanged for a methylene bromide group. Fig. 1 shows the mass spectra of $MeHgCH_2Br$ and $PropHgCH_2Br$. As can be seen, the fragmentation patterns were similar. Typical clusters of molecular ions (*m/z* 306–314 and 334–342) were observed owing to the natural isotopic composition of bromine and mercury. The ions *m/z* 310 and 338 had the highest relative abundances of 30 and 45%, respectively. The split-off of alkyl groups gave the same $HgCH_2Br^+$ cluster (*m/z* 291–299) in both spectra. Also, the fragments $HgBr^+$ (*m/z* 277–285) and Hg^+ (*m/z* 198–204) were found in both spectra. The $PropHg^+$ fragments (*m/z* 241–247) were seen in the $PropHgCH_2Br$ spectra only. The fragment cluster *m/z* 212–219 contains CH_3Hg^+ and CH_2Hg^+, with different relative abundances and patterns for the two compounds. The most abundant fragments in both spectra were CH_2Br^+ (*m/z* 93, 95).

Mass-selective detection. In the cluster of molecular ions in the $MeHgCH_2Br$ spectra, the ion with the highest abundance, *m/z* 310, represented only *ca.* 26% of the ions. However, since several mercury-containing fragment ions were obtained, it was possible to choose ions which were analytically more favorable. Thus, *m/z* 295, 215 and 202 also showed negligible interferences from the sample matrix and column (Fig. 2). The fragment *m/z* 215 was monitored because of the better signal-to-noise ratio at low concentrations. In the case of $PropHgCH_2Br$, the molecular ion *m/z* 338 was monitored because it had the highest sensitivity and negligible interferences, but fragments *m/z* 295, 281 and 202 were also satisfactorily free from interferences (Fig. 2).

The selectivity can be increased when two or more molecular ions or fragments are monitored simultaneously. The ratios between the above-mentioned fragments were thus examined to ensure peak identity and peak purity. In the case of MeHg, the relative standard deviation (R.S.D.) of the ratio between the *m/z* 215 and the other fragments was 4–5%. When monitoring PropHg, the standard deviation of the ratio between the

TABLE I

LEVELS OF TOTAL MERCURY, INORGANIC MERCURY AND METHYLMERCURY IN SAMPLES OF BLOOD CELLS AND WHOLE BLOOD

Sample[a]	Total mercury[b] (μg/l)	Inorganic mercury[b] (μg/l)	Methylmercury[c] (μg/l)
Blood cells			
Subject 1	3.9 (3.7–4.0)	0.4 (0.3–0.4)	3.5 (3.4; 3.6)
Subject 2	2.4 (2.2–2.5)	0.6 (0.4–0.7)	2.9 (2.9; 2.9)
Subject 3	2.2 (2.0–2.4)	0.6 (0.6–0.6)	1.8 (1.7;1.9)
Subject 4	16.2 (15.7–16.7)	0.4 (0.3–0.4)	17[d] (16; 18)
Subject 5	28.3 (27.61- 29.2)	0.5 (0.4 -0.7)	35[d] (33; 37)
Subject 6	13.9 (13.3–14.4)	0.6 (0.6–0.6)	17[d] (16; 18)
Whole blood			
Subject 7	6.4 (6.4–6.5)	4.9 (4.8–5.0)	1.3 (1.3; 1.4)
Subject 8	6.3 (6.3 6.3)	4.7 (4.6 4.9)	0.7 (0.7; 0.7)
Subject 9	8.3 (7.7–9.0)	6.0 (5.4–6.6)	1.6 (1.6; 1.6)
Subject 10	7.7 (7.5–7.8)	5.8 (4.5–6.6)	2.1 (1.9; 2.3)
Seronorm 904	3.4 (3.2–3.5)	1.0 (1.0–1.0)	3.4 (3.2; 3.6)

[a] Subjects 1–3 were not fish consumers, 4–6 were high fish consumers while subjects 7–10 had a normal fish consumption and a slight, occupational exposure to mercury vapour.

[b] Average and range of three determinations.

[c] Duplicate analysis.

[d] Extrapolated values within the linear range but above the concentrations of the analytical standards in the sample sequences.

molecular ion and the molecule fragments was 6–12%.

Quantitative analysis

Calibration graphs. Human blood samples were spiked with MeHgCl and the work-up procedure was performed. For each concentration, two sample preparations were made. The calibration graphs were linear and passed virtually through the origin for the concentration range 1–30 μg/l, when the background was subtracted. The calibration graph for the concentration range 1–5 μg/l gave a correlation coefficient of 0.992 (n = 12). No noticeable differences were found for peak-height and peak-area measurements.

Precision. The usefulness of PropHgBr as an internal standard is demonstrated by the high precision in the GC–SIM analysis at low levels. Ten different preparations of a blood sample containing *ca.* 2 μg/l and of the same sample spiked with 8 μg/l were analyzed. The R.S.D. was 4.2 and 5.5%, respectively. When working up and analyzing a blood sample containing *ca.* 2.9 μg/l mercury on three different days, the standard deviation was 9%.

Detection limit. The detection limit, calculated according to Miller and Miller [21], for MeHg in human blood was *ca.* 0.5 μg of mercury per liter of blood.

Comparison with levels of total mercury and inorganic/ionic mercury levels. Inorganic mercury added to whole blood and blood cells was recovered with the same sensitivity as from aqueous standards (the same slope of the calibration curve). No interference was detected from added MeHg (less than 1% decomposition of 60 μg added). The detection limit for inorganic mercury and total mercury in blood was *ca.* 0.2 and 0.5 μg/l, respectively. The difference is explained by the application of an equilibration step between the sample solution and the gas phase (20 s shaking) prior to the aeration into the gas cell, thus increasing the peak height in the method for inorganic mercury.

Table I shows the results of the analysis of total mercury, inorganic mercury and MeHg in blood cells from subjects with different fish consumption, and in whole blood from subjects occupationally exposed to mercury vapor. A reference sample of lyophilized blood (Seronorm Batch 904, Nycomed, Oslo, Norway) was also analyzed. The "recommended" value of the reference sample was 4.0 μg/l (20 nmol/l), and the mean and range obtained by five different laboratories were 3.8 and 2.0–6.0 μg/l.

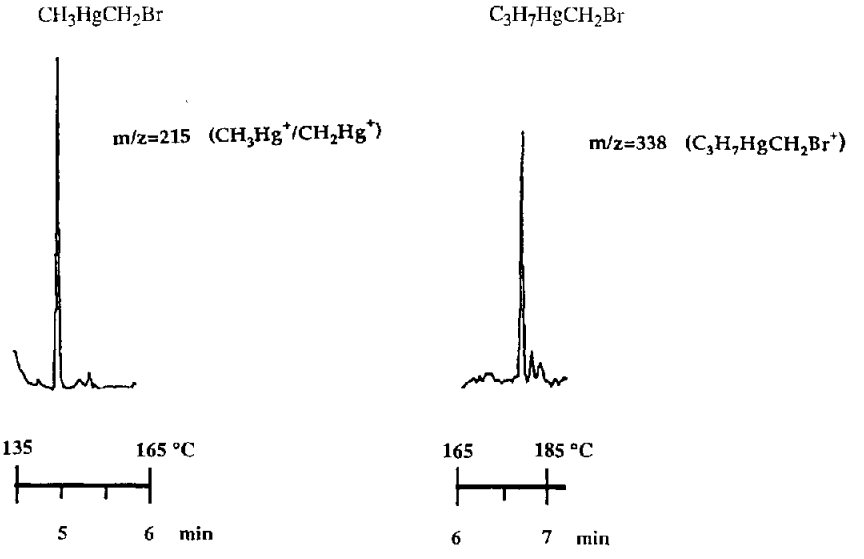

Fig. 3. Selected-ion monitoring of a blood sample from a fish consumer containing *ca.* 15 μg/l methylmercury and 12.5 μg/l propylmercury (internal standard). Conditions: see Fig. 2.

Applications

The method is applicable for the monitoring of MeHg in human blood. The potential of the method is illustrated by the high selectivity and sensitivity. Mass fragmentograms of a blood sample containing *ca.* 15 μg/l, from a fish consumer, are shown in Fig. 3.

CONCLUSIONS

The sensitivity and the selectivity of high-resolution GC–SIM is demonstrated to give precise determination of MeHg in blood. The use of PropHg as an internal standard and derivatization with diazomethane increases the precision and sensitivity of the method.

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