Journal of Chromatography, 97 (1974) 65–70

CHROM. 7540

DETERMINATION OF METHYLMERCURY IN BLOOD BY GAS CHROMATOGRAPHY

R. VON BURG*, F. FARRIS** and J. C. SMITH***

Department of Pharmacology and Toxicology, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642 (U.S.A.)

(Received January 21st, 1974)

SUMMARY

A sensitive gas chromatographic method for the determination of methylmercury concentrations in milliliter samples of whole blood is described. The method employs oxalic acid as a hydrogen ion source, potassium iodide as a halide ion source, sodium hydroxide to remove unwanted impurities and radioactive ^{203}Hg for the precise calculation of recovery. Ethylene glycol succinate (2%) on a solid support of Chromosorb G or Chromosorb T was used as column packing. The advantages and disadvantages of both these packing materials are discussed.

INTRODUCTION

For some time we have employed gas chromatography for the determination of methylmercury in biological materials. There are a number of published methods for the measurement of methylmercury in samples where its concentration is of the order of 0.1 μg/g of sample; for such samples, we have normally used a simple modification of the Westöö method[1]. This proved to be adequate until we attempted to extend it to the measurement of methylmercury in blood of normal populations. By a series of modifications we can now make reliable estimations of methylmercury in samples containing as little as 1 ng. Although the method was originally developed for application to blood, it is also applicable to most other biological materials.

EXPERIMENTAL

Materials

The following material was used: Oak Ridge tubes, polypropylene, capacity 50 ml; each tube was discarded after a single extraction. Pyrex glass tubes (16 × 150 mm) equipped with PTFE-lined screw caps. Disposable glass pipettes of capacity 1 ml and 2 ml obtained from Kimble Glass, Inc.

* Present address: Department of Biology, Ramapo College, Mahwah, N.J. 07430, U.S.A.
** Present address: Toxicology Laboratory, Memorial Hospital, Springfield, Ill., U.S.A.
*** To whom requests for reprints should be addressed.

The following reagents were obtained from Fisher Scientific Co., Fair Lawn, N.J., U.S.A.: benzene, spectroanalyzed; urea, A.C.S. certified; oxalic acid, certified, anhydrous; potassium iodide, A.C.S. certified; 30% ammonium hydroxide, A.C.S. reagent grade.

L-Cysteine (free base) was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.; high-purity nitrogen (dry) from Linde Division, Union Carbide Corporation, New York, N.Y., U.S.A.; and methylmercury chloride from K and K Laboratories, Inc., Plainview, N.Y., U.S.A. The methylmercury salt was recrystallized from 100% ethanol before use.

Methyl[^{203}Hg]mercury chloride was obtained from New England Nuclear, Boston, Mass., U.S.A.; sepcific activities were in the range of 1.5 to 5.0 mCi/mg of mercury. If necessary, this material was purified by extraction into benzene.

Methods

All columns used were 6 ft. by 4 mm I.D. coiled glass. The columns may be packed with either of two packing materials that we have found to be suitable for methylmercury separation and analysis. The first of these is a liquid phase of 2% ethylene glycol succinate (EGS) on a solid support of Chromosorb G (AW-DMCS) 60–80 mesh (Hi-Eff 2B, Applied Science Labs., State College, Pa., U.S.A.). The second packing material is Chromosorb T (PTFE), 40–60 mesh (Applied Science). Both materials may be packed by the same basic method. The Hi-Eff 2B, however, is more convenient in that it flows freely and may be used easily at room temperature. The Chromosorb T, on the other hand, should be stored in a freezer and packed in a cold room at 0–5°. Packing material is added with the aid of vibration and vacuum until all but about 10 cm of the column has been filled. A small plug of quartz wool is placed directly over the packing, and approx. 6 cm of solid, ground potassium iodide sifted through an 80-mesh screen, is put on top of this plug and secured by another quartz wool plug. The columns are conditioned for 36 h at 30° above the normal operating temperature with a nitrogen flow-rate of 20–40 ml/min in the Packard, Model 7401, gas chromatograph. Detectors are left disconnected during the conditioning period.

After conditioning, the detector is connected and the chromatograph is adjusted to normal operating conditions. For the Hi-Eff 2B column, the detector and inlet temperatures are set at 180° while the oven is maintained at 150°. The gas flow-rate is 100–120 ml/min under a pressure of 36 p.s.i. The Chromosorb T column is operated at 100° for the inlet and detector, with the oven set at 70° and the gas flow-rate at 80 ml/min. Usually, an additional period of 24 h is required to establish equilibrium and a constant baseline. A series of injections of a methylmercury halide standard should complete the column preparation. It seems that, normally, 5–10 of these injections (1–5 ng per injection) are sufficient, but it is advisable to continue the injections of standard until a constant peak height is obtained. The electron-capture detector (150-mCi tritium foil) is operated at a suppression current of 10^{-7}–2×10^{-7} A at a potential of 3–7 V.

Sample preparation. The sample (normally 1.0 ml) of heparinized whole blood is placed in a 50-ml polypropylene Oak Ridge tube, and a 10-μl aliquot of spike (a solution containing less than 1 ng of methyl[^{203}Hg]mercury, approximately 3000 dpm) is added by means of an Eppendorf pipette. The spiked blood is mixed and

allowed to stand for 15 min, then 8 ml of 8 *M* urea, 2 ml of 1 *M* oxalic acid, 1 ml of 1 *M* potassium iodide and 15 ml of benzene are added and the tube is capped and shaken for 10 min on a Thomas–Boerner shaking apparatus; it is then centrifuged at 15,000 *g* for 10 min in a Sorval superspeed centrifuge. The benzene layer is transferred with Pasteur pipettes to a second Oak Ridge tube, and another 15 ml of benzene are added to the sample, which is shaken and centrifuged as before; the two benzene layers are combined. To the tube containing the 30 ml of benzene is added 1 ml of 0.1 *M* sodium hydroxide, then the tube is capped, shaken for 5 min and centrifuged for 5 min at 3000 *g*. The sodium hydroxide layer is removed with Pasteur pipettes and discarded. To the washed benzene is added 1 ml of 1.5% alkaline cysteine solution (adjusted to pH 10 with ammonium hydroxide), and the tube is capped, shaken for 10 min and centrifuged at 3000 *g*. The cysteine layer is removed and placed in a 15-ml glass tube equipped with a PTFE-lined screw cap. To this tube are added 1 ml of 1 *M* oxalic acid, 1 ml of 1 *M* potassium iodide and 1 ml of benzene, and the tube is capped, mixed in a vortex mixer for 10 min and centrifuged at 3000 *g* for 5 min. The benzene layer is then removed by Pasteur pipette and placed in a small culture tube, and this tube is corked. Finally, this benzene extract is counted in either a Packard Model 3002 Auto-gamma spectrometer, or in a Packard Tri-Carb liquid scintillation spectrometer.

Analysis and calculations. A standard curve is established for the present conditions of the column before each day's determinations. Depending on the status of the column, the methylmercury peak appears 1.5 to 2 min after injection on the EGS column and after about 30 sec on the PTFE column. The amount of methylmercury per injection is determined by reading the height of the peak directly from the chart paper and comparing the peak height of the unknown sample with the peak heights of the standard curve. The percentage recovery for each extraction is determined by measuring the cpm in the final benzene solution in comparison with that for the 10 μl of added spike. Relevant calculations for the method are as follows:

$$\text{Recovery of methylmercury, \%} = \frac{\text{cpm in final benzene} \times 100}{\text{cpm in 10-}\mu\text{l spike}} \quad (1)$$

$$\begin{aligned}\text{Total weight of methylmercury in sample, ng} &= \text{methylmercury injected, ng}/\mu\text{l} \\ &\quad \cdot \frac{1000\ \mu\text{l}}{1\ \text{ml}} \cdot \frac{1\ \text{ml} \times 100}{\text{recovery, \%}} \\ &= \frac{\text{methylmercury injected, ng}}{\text{recovery, \%}} \cdot 10^5 \end{aligned} \quad (2)$$

$$\text{Methylmercury content in sample, ppb} = \frac{\text{total weight of methylmercury in sample}}{\text{weight of sample, g}} \quad (3)$$

RESULTS AND DISCUSSION

We have successfully developed a system for the analysis of methylmercury in blood. In order to do so, it was necessary to make some rather intricate modifi-

cations of earlier methods. Regardless of how trivial some of these changes may appear, no alterations were incorporated unless they were found to improve significantly the over-all method.

There are several methods for measuring methylmercury in fish and tissue samples where levels are of the order of 0.1 ppm. An original attempt to apply such a method to the determination of methylmercury in blood led to low recovery. Since concentrated urea solutions tend to uncoil proteins, it was thought that such treatment might increase methylmercury recoveries. Theoretically, uncoiled proteins would expose more of the methylmercury-binding sites for extraction; therefore, 8 *M* urea was used as the hemolyzing agent, rather than distilled water. Although recoveries were not greatly increased, the initial benzene extract from blood was much cleaner, and on this basis the use of urea was continued. For samples other than blood, however, water is still the preferred medium for homogenization.

In spite of the assistance of urea, the benzene after extraction contained numerous colored impurities. Interestingly, the brown impurities were carried through the remainder of the extraction procedure along with the methylmercury. From the first benzene solution they were extracted into the alkaline cysteine solution, and, on acidification of the cysteine layer, the impurities passed to the final benzene extract. The apparent affinity of the impurities for alkaline aqueous solutions suggested the use of an alkaline wash, and 1 ml of 0.1 *M* sodium hydroxide was found to remove practically all the interfering impurities. Unfortunately, a max. of about 10% of the methylmercury present was lost in the alkaline wash.

Mineral acids were an additional source of contamination. Commercially prepared halogen acids are routinely used for extraction of methylmercury from such biological samples as fish, but benzene extracts of hydrochloric, hydrobromic and hydriodic acids yield small interfering peaks on the gas chromatogram, especially at high sensitivity settings. In addition to interfering peaks, these acids give extensive late peaks. Commercially available mineral acids were thus unsatisfactory, and the use of a high-purity organic acid to adjust the pH seemed reasonable, as long as a source of halide ions was added to allow extraction of the methylmercury. Oxalic acid was chosen, primarily because of its insolubility in benzene, which prevented extraction of the acid into the benzene layer. Potassium iodide was superior to sodium chloride as a halide source in that it led to better recoveries; the use of sodium iodide was not attempted. Use of oxalic acid and potassium iodide as acid and halide sources has, in our hands, increased the average recovery by as much as 10%.

The extraction of methylmercury as the iodide form necessitates the measurement of standards in the iodide form as well. Consequently, a pre-pack of a few centimeters of finely ground potassium iodide was incorporated into all columns. Methylmercury chloride is presumably converted into the iodide form by ion exchange on the column; thus, the more stable methylmercury chloride can be used in standard solutions.

Usually, 80 to 90% of the methylmercury in the sample is extracted with the first portion of benzene, and the second benzene extraction removes the remainder. As previously stated, the sodium hydroxide wash removes about 10% of the methylmercury. The cysteine extraction effectively removes all the methylmercury from the benzene, but large recovery losses can be encountered during the final step. The addition of cadmium chloride or mercuric chloride to the cysteine to occupy the ex-

cess –SH binding sites may possibly facilitate the partition of methylmercury into the final extract, but the effect is not marked with the current protocol.

When dealing with volunteers or patients, the smallest workable sample of blood is desirable. Usually, 1 ml is enough for this method, but the sample size may easily be extended to 2 or 3 ml, and even 5-ml samples can be handled with little modification of the method.

Although recovery rates are improved by this method, the large number of steps and the extensive handling of the samples required for this type of procedure make partial recoveries almost inevitable. Recovery rates from blood average 60 to 70% on routine samples, but have reached as much as 95%. The potential scatter is large, and in consequence it was necessary to find a technique that allowed suitable corrections. Monitoring with methyl[^{203}Hg]mercury solved this problem, and periodic evaluation of each extraction step was possible. Normally, less than 1 ng is added to the sample before extraction. The specific activity is such that this amount corresponds to at least 3000 dpm. For samples of very low methylmercury content correction is made for the added spike.

The EGS columns, once conditioned and functioning properly, have proved somewhat superior to Chromosorb T in routine use. They are capable of absorbing large amounts of discolored or water-saturated solutions without extensive loss of sensitivity, and they generally give better peak separation than does PTFE. The PTFE columns, on the other hand, have several advantages over the EGS columns. Retention times are shorter, retention of methylmercury being approximately 30 sec as compared with 1.5 to 2 min for the EGS columns. Whereas EGS columns can handle only 2000 to 3000 sample injections before replacement becomes necessary, the PTFE columns may last indefinitely with cautious treatment and may be removed from the oven without deleterious effects. At optimal operating conditions, either column allows detection and measurement of organomercurials in blood samples from normal populations, but PTFE has the potential of providing twice the sensitivity of EGS. However, if the balancing current is not above 10^{-7} A at the start of the analysis, the foil and the anode probe must be cleaned with ferric oxide or replaced.

It is important to emphasize that the extraction procedure is not specific for methylmercury, but works equally well for other organomercury compounds: indeed, ethylmercury is detectable at somewhat lower levels than methylmercury. In addition, although the method was developed for application to blood, it is equally suitable for measurement of organomercury compounds in any biological sample that lends itself to a benzene extraction. It has been successfully applied to wheat, fish and a variety of other tissue samples, and comparison with the values for "organic" mercury obtained as the difference between total and inorganic mercury as determined by atomic absorption shows good correspondence. In samples from a number of populations studied jointly with Dr. T. W. Clarkson[2], it appears that the amount of methylmercury determined by gas chromatography is sufficient to account for all organic mercury in the blood.

ACKNOWLEDGEMENT

This work was supported by grants GM-015190 and GM-01781 from the National Institutes of Health and a grant from the National Science Foundation (RANN: GI-300978).

REFERENCES

1 G. Westöö, *Acta Chem. Scand.*, 22 (1968) 2277.
2 T. W. Clarkson and M. R. Greenwood, *Anal. Biochem.*, 37 (1970) 236.