

THE DETERMINATION OF SMALL AMOUNTS OF MERCURY IN BIOLOGICAL MATERIAL

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A simple method is described for the digestion of biological material containing mercury. The procedure is based on complete volatilization of mercury and subsequent absorption in an aqueous solution of potassium permanganate and sulphuric acid. Mercury ions are determined with dithizone using the reversion method. The statistical treatment of the calibration line shows that the proposed method is sufficiently sensitive and precise for toxicological work.

The determination of mercury in biological material involves the handling of small quantities of mercury in the presence of large amounts of organic matter. The digestion of biological material by the usual methods brings about considerable loss of mercury (1, 2, 3, 4). Some authors (5) consider the analysis of mercury in organic material an illusory task so long as the problem of the digestion is unsolved. Thus, the problem of the determination of mercury in biological material lies in the digestion procedure rather than in the analytical method for the final determination of mercury.

Experimental

Reagents

Potassium chlorate, p. a. Schering-Kahlbaum, 5% solution in 0.25 N hydrochloric acid. Traces of metals were removed by shaking with the solution of dithizone in chloroform. *Sulphuric acid*, p. a. Malincrodt, St. Louis 94%. By appropriate dilution a 0.25 N solution was prepared and purified with a dithizone solution in chloroform. *Potassium permanganate* p. a. E. Merck, Darmstadt. 0.01 M and 0.1 M solution were prepared in 0.25 N sulphuric acid.

Chloroform. The purity of chloroform used for the preparation of dithizone solutions is of major importance. Even the slightest amounts of oxidizing agents affect the stability of dithizone solutions. Chloroform

should, therefore, be purified before use and stored in a cold and dark place (possibly in a refrigerator) until it is used. Dithizone solutions should be stored in the same way.

Various methods are recommended for the purification of tetrachloromethane and chloroform (6, 7, 8, 9). After a series of experiments the following procedure has proved satisfactory for the purification of these solvents.

Distil either pure commercial p. a. or used chloroform on a water bath, adding 20 ml of 20% aqueous sodium hydroxide solution and some crystals of sodium thiosulphate per 1 l of chloroform until about 100 ml remain in the distillation flask. By this first distillation only the heaviest impurities are removed. Redistil this chloroform adding 20 ml of 20% sodium hydroxide solution and some crystals of sodium thiosulphate. Distil the distillate obtained once again adding a few crystals of sodium thiosulphate and dry it using plenty of dry sodium sulphate. Filter the liquid and perform a vacuum distillation (water suction pump) at 33–34° C. The collector should be wrapped up in ice, and the whole apparatus should be protected from daylight. 1–1.5% of redistilled ethanol should be added to the pure chloroform for preservation (10).

Dithizone solutions prepared from chloroform purified in this way have proved to be stable for 4 months if stored in a refrigerator at +2° C. The stability was checked by measuring the extinction of the same solution at different time intervals.

Reversion solution. Dissolve 20.4 g biphthalate and 50 g of potassium iodide in 1000 ml of redistilled water and add a few drops of a diluted solution of sodium thiosulphate to reduce the free iodine. Shake the solution with a few portions of the dithizone solution, until the last portion remains green. Keep the solution in a cold, dark place to prevent the formation of free iodine, as the presence of the slightest amount of it will decompose the dithizone.

10% *hydroxylamine sulphate* solution. Weigh 10 g of $\text{NH}_2\text{OH} \times \frac{1}{2} \text{H}_2\text{SO}_4$, dissolve in 100 ml of 0.25 N sulphuric acid and shake with the dithizone solution, until the last extract remains unchanged.

Mercury standard solution. Dissolve HgCl_2 p. a. in 0.25 N HCl. The standards of lower concentrations (1 μg Hg/ml) were obtained by diluting the concentrated solutions containing 200 μg Hg/ml by 0.25 HCl. Only fresh standards were used to avoid possible adsorption on glass.

Dithizone. Dissolve about 50 mg of dithizone (diphenylthiocarbazone, J. D. Riedel–E. de Haen) in 25 ml of chloroform and filter the solution through a coarse sintered glass filter to remove any insoluble material. Transfer the dithizone solution into a one-litre separating funnel containing 500 ml of 2% ammonia. By vigorous shaking of the dithizone solution with the aqueous ammonia solution, dithizone is transferred

into the aqueous layer in the form of ammonium dithizonate. All impurities, particularly diphenylthiocarbodiazone (11, 12), a brown oxidation product of dithizone, remain in the organic layer. Separate the two phases and shake the aqueous one several times with small portions of pure chloroform. Repeat this procedure several times, until the brown colour indicating impurity disappears from the organic phase. Make the ammoniacal solution slightly acid with redistilled hydrochloric acid to precipitate dithizone. Extract the finely dispersed dithizone with small portions of chloroform until all the dithizone is transferred into the organic phase. Transfer this pure dithizone solution into a clean separating funnel and wash it several times with redistilled water until a neutral reaction is obtained for the aqueous layer. Store concentrated dithizone solution in chloroform in a dark glass bottle and dilute it with chloroform as needed.

Water. Redistilled water (glassware Pyrex) should be used throughout the procedure. Its purity should be checked frequently with dithizone.

Hydrochloric acid, concentrated, p. a. Merck, Darmstadt, redistilled 20%.

Nitric acid concentrated, of analytical purity, Malincrodt, St. Louis, redistilled.

Ammonia, concentrated, of analytical purity, Malincrodt, St. Louis, redistilled.

Apparatus

A Beckman Model DU spectrophotometer with matched 10.0 mm Corex cells was used for all spectrophotometric measurements; a tungsten lamp served as the light source. Extinction values were obtained by using a »red sensitive« (cesium oxide) phototube. The values of the extinction were the arithmetic means of three subsequent measurements. The nominal spectral band width was 3 m μ .

Measurement of the hydrogen ion concentration was carried out with a Beckman Model M pH Meter.

An International Centrifuge Model E-220 was used at 3000 r. p. m. for centrifugation operations.

Separating funnels were shaken by hand, at 130 strokes per minute.

The procedure concerning the cleaning of glassware was as follows: After being kept for a fairly long time in chromic sulphuric acid, glassware was first rinsed with warm tap water and then with distilled water. It was then washed with hot 10% sodium hydroxide solution, with distilled water and hot nitric acid (1:2), and finally with redistilled water.

All glassware was of Pyrex or Jena glass. Separating funnels were pear-shaped (Squibb).

Digestion of biological material

The digestion of biological material can be performed either by dry procedures with or without oxidizing agents, or by wet procedures using various chemicals, primarily acids and oxidizing agents. Both types of digestion, however, require the heating of the sample, the dry procedures up to 500–550° C, the wet ones at least up to the boiling point of the least volatile acid. A number of the existing digestion procedures for analytical purposes have recently been reviewed by Middleton and Stuckey (13).

We attempted first to digest blood and urine by using nitric acid and hydrogen peroxide. This method has previously been described (14) and used in our laboratories with good results. However, when applied to pure solutions containing only mercury ions, we found that it gave results which were not reproducible; they showed no regularity, differing from one experiment to another a great deal. Very often the amount of mercury found after digestion was less than 10 percent of the amount added. The assumption that this was due to the volatilization of mercury in the course of digestion was confirmed by the fact that boiling with a reflux condenser gave far better and higher results. Yet, the loss of mercury could not be prevented in this way.

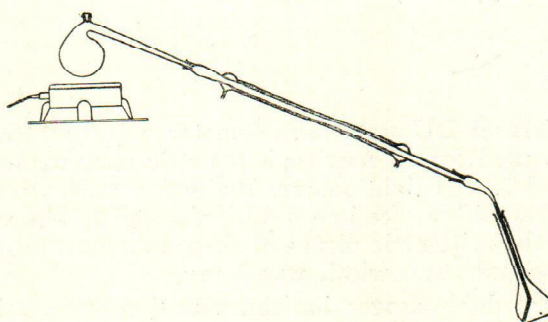


Fig. 1

Another procedure was, therefore, devised by taking advantage of the volatility of mercury and its compounds. The new procedure consisted in »distilling« mercury from the organic material into an absorption solution containing potassium permanganate. A simple apparatus was constructed which served both for the acid digestion and the distillation of mercury. As seen from Fig. 1, the apparatus consisted of a retort (a), a condenser (b), and an attachment (c). The distillate was collected in a 100 ml volumetric flask containing 10 ml of aqueous solution of potassium permanganate (0.01 M) and sulphuric acid. Owing to its oxidizing properties this solution retained mercury ions, as well as the

elementary mercury from the air-vapour mixture bubbling through it. The digestion of the organic matter was effected by potassium chlorate and hydrochloric acid. The presence of chloride ions proved to increase the rate of the volatilization of mercury.

Determination of mercury

A dithizone method using the reversion principle was chosen for the final determination of mercury. The reversion principle was first described by H. Fischer (15), and discussed more fully by Irving and co-workers (16, 17). This method seems to be particularly suitable for the determination of mercury in biological material where mercury is always accompanied by copper. Since copper ions behave in relation to dithizone in a similar way to mercury ions, they interfere with the determination of mercury. If a suitable reversion agent is used which only reverts the mercury dithizonate leaving the copper dithizonate intact, the reversion value $R = E_r - E_m$ will be proportional to the concentration of mercury ions only, and copper ions and other impurities will not affect the result.

In the equation for the reversion value E_r denotes the extinction of the solution containing dithizone after the reversion of mercury dithizonate to mercury ions and dithizone, while E_m is the extinction of the mixed colour solution containing mercury dithizonate, excess of dithizone, and various coloured impurities. In our experiments an acid solution of potassium iodide was used as the selective reversion agent (18). The reversion method is most sensitive if the extinction is measured at the wave length for which the difference ($2 \epsilon_r - \epsilon_k$) is greatest (ϵ_r, ϵ_k being the molar extinction coefficients of dithizone and mercury dithizonate respectively). For mercury this condition is satisfied by choosing the wave length of maximum absorption of dithizone in chloroform (608 $m\mu$), the absorption of mercury dithizonate at this wave length being very small.

According to Irving (18), the presence of chlorides interferes with the determination of mercury by dithizone. If the digestion is carried out with potassium chlorate and hydrochloric acid in the described way, the absorbing liquid will contain considerable quantities of dissolved chlorine, chlorides, and other chlorine compounds, as well as a considerable amount of free potassium permanganate which would immediately oxidize dithizone into inactive diphenylthiocarbodiazone (11, 12). To avoid this, potassium permanganate, dissolved chlorine and other chlorine compounds were reduced by hydroxylamine-sulphate, the sulphate being used to avoid the unnecessary increase in the concentration of chlorides. Our experimental results proved that the amount of chlorides present in the absorbing fluid did not interfere with the determination of mercury by the reversion method when the excess of the

permanganate ions had been removed by reduction with hydroxylamine. Thus, it seems that the determination of mercury by the reversion method can be performed even in the presence of moderate amounts of chlorides.

Calibration curve

The procedure to establish the calibration curve consisted of four independent series of experiments.

In the first series of experiments the separating funnel was filled with x ml of mercury standard solution containing $1 \mu\text{g Hg/ml}$ in 0.25 N HCl and $(20-x)$ ml 0.25 N HCl , 25 ml of redistilled water, 20 ml of 0.01 M KMnO_4 , 20 ml of $\text{NH}_2\text{OH} \cdot \frac{1}{2} \text{H}_2\text{SO}_4$ and 6 ml HCl (redistilled). The composition of this solution was analogous to the absorbing liquid containing the distillate.

In the second series of experiments there were placed in the retort x ml of mercury standard solution (as in the first series), $(20-x)$ ml of 0.25 N HCl , 5 ml of potassium chlorate solution, $5 \text{ ml H}_2\text{O}$ and $2 \text{ ml } 20.2\%$ of HCl (redistilled); 10 ml of 0.01 M potassium permanganate solution was put in the collector. The contents of the retort were evaporated to dryness by careful heating. After cooling 3 ml of KClO_3 and 2 ml HCl were added. The same quantities of potassium chlorate and hydrochloric acid were added once again, allowing the retort to be heated for a further hour and a half. The tube of the retort, the condenser and the attachment were washed with a total of 10 ml of potassium permanganate solution. The contents of the collector were transferred into a separating funnel and 20 ml of $\text{NH}_2\text{OH} \cdot \frac{1}{2} \text{H}_2\text{SO}_4$ solution were added.

In the third series of experiments the procedure was the same as in the second series, except that instead of 5 ml of water 5 ml of blood were added together with 5 glass beads. The blood used in this series of experiments was the same throughout. After second heating, addition of 1 ml of a 0.1 M potassium permanganate solution in the collector was needed, as the disappearance of the violet colour indicated the absence of potassium permanganate.

In the fourth series of experiments 25 ml of urine were added instead of blood. The urine used in this series of experiments was the same throughout.

The solutions prepared in this way were transferred into separating funnels and shaken with 20 ml of dithizone solution in chloroform. The extinction was measured at $608 \text{ m}\mu$. Its value was about 0.500 for all the concentrations of mercury used.

After allowing the layers to separate, half of the organic phase (cca 10 ml) was placed into a centrifuge tube, while the other half was transferred into another separating funnel, and 10 ml of the reversion

solution were added. After shaking, the organic phase was separated and transferred into another centrifuge tube. After being centrifuged the contents of the tube were transferred into the spectrophotometric cells and the extinction was measured at 608 $m\mu$. E_m value was obtained by measuring the extinction of the solution contained in the first tube, and E_r value of the solution contained in the second centrifuge tube.

4 blanks were run in each series of experiments.

Both the first and second extraction lasted 3 min. This was checked by special tests.

Results and discussion

Reversion values for the three series of experiments (corrected for blanks and multiplied by 1000) are shown in Table 1. Each reversion value (R) in the table is the mean of two parallel independent determinations; subscripts indicate the series of the experiments performed.

Table 1

Data used in establishing the calibration curve for the determination of mercury in biological material

C	R ₁	R ₂	R ₃	R ₄
1	18	16	11	11
3	58	54	49	54
5	88	89	89	94
7	124	132	117	125
9	164	172	162	164
11	201	210	199	206
13	236	234	223	241
15	269	269	265	277
17	294	294	282	298

The results of the statistical analysis are given in Table 2, for each series separately. All four regression lines pass through the origin. By inspection of Table 2 it can be seen that the regression coefficients do not differ much. It has, therefore, been assumed that all the samples belong to a population with the same regression coefficient, the differences being due to the sampling error.

Table 2
Linear regression analysis of the calibration curve

Series of experiments	$b \times 10^3$	$s_b \times 10^3$	1/b	$s_{1/b}$	$s \times 10^3$
I	17.8	0.1	56.1	0.5	4.500
II	17.7	0.2	55.4	0.7	7.331
III	17.3	0.2	57.9	0.7	6.605
IV	18.2	0.2	55.0	0.6	5.711
Common regression line	17.8	0.1	56.1	0.3	6.933

The usual procedure for testing this hypothesis was used (19). The results of the analysis of variance are given in Table 3.

Table 3
Analysis of variance

Source of variation	Sum of squares	Degrees of freedom	Variance
About common regression . . .	16.82×10^{-4}	35	
Total	12.01×10^{-4}	32	3.752×10^{-5}
Difference	4.81×10^{-4}	3	1.604×10^{-4}

They prove that the hypothesis chosen was correct. Thus, for the estimation of the concentration of mercury in any of the four materials a common equation can be used:

$$c = (56.1 \pm 0.3) R \mu\text{g Hg/ml CHCl}_3 \quad (1)$$

The regression line and the 95% confidence limits for the common regression line are presented in Fig. 2.

Thus, a calibration line obtained with a pure solution of mercury ions can be used for the determination of mercury in biological material.

One more advantage of the method described is that the final determination is performed in a comparatively pure system whose composition does not change essentially from one analysis to another. This in no small measure adds to the precision of the method.

The confidence limits for the estimation of the concentration of mercury from the measured reversion values indicate that the method is reliable and sufficiently precise for toxicological work. If the concentrations of mercury ions per 20 ml of CHCl_3 are converted to the concentrations of mercury in blood or urine, under the assumption that for the determination 5 ml of blood or 25 ml of urine were used, a con-

centration of $10 \mu\text{g Hg}/20 \text{ ml CHCl}_3$ corresponds to the concentration of $200 \mu\text{g Hg}/100 \text{ ml}$ of blood and $40 \mu\text{g Hg}/100 \text{ ml}$ of urine, respectively. The standard error, which in the concentration range of $20 \mu\text{g Hg}/20 \text{ ml CHCl}_3$ is $0.4 \mu\text{g Hg}$, does not amount to more than $8 \mu\text{g}$ in the concentration range of $350 \mu\text{g Hg}/100 \text{ ml}$ of blood, and $1.6 \mu\text{g Hg}$ in the concentration range of $68 \mu\text{g Hg}/100 \text{ ml}$ of urine. This precision is satisfactory both for industrial-hygiene purposes and for toxicological investigations.

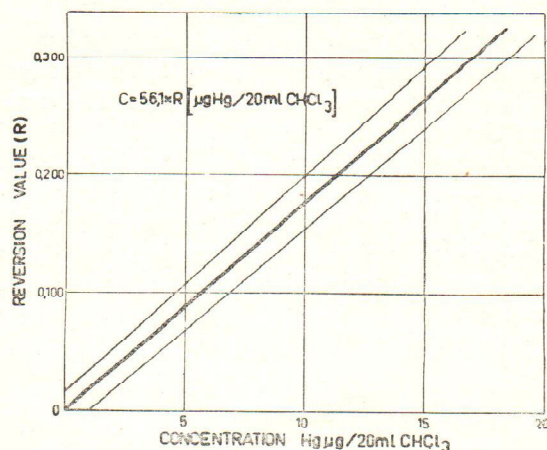


Fig. 2

Recommended procedure

On the basis of these results, the following procedure is proposed for the analysis of mercury in blood and urine:

Place 5 ml of blood or 25 ml of urine in the retort (Fig. 1), containing few small glass beads. Add 5 ml of a 5% potassium chlorate solution and 2 ml of 20.2% hydrochloric acid. Pour 10 ml of 0.01 M aqueous potassium permanganate solution into the collector. Heat the retort until the contents become dry, cool to room temperature and add another portion (3 ml) of potassium chlorate and 2 ml of hydrochloric acid. If the violet colour of the absorbing solution in the collector disappears, add 1 ml of 0.1 M potassium permanganate. Heat the retort again to dryness and cool. Add the same amount of potassium chlorate and hydrochloric acid again and heat the retort for about 90 min. After cooling, wash the retort tube, the condenser and the attachment with 10 ml of a 0.01 M potassium permanganate solution. Transfer the contents of the collector into a separating funnel, add 20 ml of an aqueous so-

lution of hydroxylamine sulphate solution and exactly 20 ml of a solution of dithizone in chloroform. Shake vigorously for 3 minutes. The extinction of the dithizone solution, measured at $608\text{ m}\mu$, should be about 0.500. After allowing the phases to separate, transfer one half of the organic phase into another separating funnel, add 10 ml of the reversion solution and shake for 3 minutes. Transfer both the remainder of the organic phase from the first separating funnel and the reverted organic phase from the second funnel into two centrifuge tubes and centrifuge both tubes at 3000 r. p. m. for 5 minutes.

The contents of the centrifuge tubes are then transferred into spectrophotometric cells of 10.0 mm light path, and the extinction measured at $608\text{ m}\mu$ using a nominal band width of $3\text{ m}\mu$. The extinction of the solution from the first separating funnel is the E_m value; the extinction of the reverted solution is the E_r value. The difference $E_r - E_m$ gives the reversion value E .

The concentration of the mercury in the sample is obtained either from a calibration curve or from the regression equation (equ. 1). The calibration curve is established with pure solutions of mercury ions of known concentrations.

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*Sadržaj*ODREĐIVANJE MALIH KOLIČINA ŽIVE U BIOLOŠKOM
MATERIJALU

Opisana je jednostavna metoda za mineralizaciju biološkog materijala, koji sadržava živu. Postupak se osniva na isparivanju žive i apsorpciji isparene žive u vodenoj otopini kalijum permanganata i sumporne kiseline. Nakon mineralizacije živini ioni se određuju pomoću ditizona (reverziona metoda). Statistička obrada baždarnog pravca pokazuje, da je predložena metoda dovoljno osjetljiva i precizna za toksikološki rad.

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