AN IMPROVED DITHIZONE METHOD FOR THE DETERMINATION OF SMALL QUANTITIES OF ZINC IN BLOOD AND TISSUE SAMPLES

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(Received for publication, July 11, 1948)

The study of zinc metabolism in experimental animals and humans is dependent upon a reliable analytical method. Numerous methods, gravimetric, turbidimetric, and colorimetric (1), have been tried and found wanting. Fischer (2) first introduced diphenylthiocarbazone (dithizone) for the analysis of metals with relation to industrial processes. This organic dye combines with a number of the heavy metals (Sandell (3)). The combination is selective for any one metal, depending upon the pH of the solution containing the metals, and the presence of salts which form complexes with other metals in solution. It was not until 1937 that Fischer and Leopoldi (4) adapted the dithizone method to the analysis of inorganic zinc. Holland and Ritchie (5) and Cowling and Miller (6) used this dye for zinc analyses in plants, and Hove et al. (7) in the measurement of carbonic anhydrase. The procedure employed by these authors, however, involved a preliminary extraction of other metals, principally copper, and a final extraction of zinc. Gettler (8) simplified the method by the use of a buffered solution of complex-forming salts, obviating the preliminary separation. We have further refined the method to obtain greater accuracy in the analysis of the zinc content of samples of whole blood, plasma, erythrocytes, and leucocytes and samples of tissue. Samples of the size which can be practicably obtained may contain as little as 1 to 5 $\gamma$ of zinc.

Diphenylthiocarbazone has the following structural formula:

$$\text{NH} - \text{NH} - C_6H_5$$

$$\text{S} = \text{C}$$

$$\text{N} = \text{N} - C_6H_5$$

The dye is soluble in chloroform and carbon tetrachloride and insoluble in water. It decomposes in aqueous alkaline solutions. When dissolved

* The studies reported herein were supported by a grant-in-aid from the National Institute of Health.
in chloroform or carbon tetrachloride, the dye is dark green in transmitted and Bordeaux red in reflected light. It does not fade on standing when stored in the dark at 4°. When exposed to sunlight the dye is oxidized, with the production of a yellow color.

At pH 5.5 and in the presence of a tartrate solution and complex-forming buffer, dithizone combines with zinc in stoichiometric proportions to form zinc dithizonate, but does not combine with other metals which may be present. The completion of this reaction is accompanied by a change of color of the dithizone from green to bright red. In routine extraction of samples this color change occurs slowly and appears to pass through an intermediary purple stage. In our experience, this color change takes place more rapidly when the dye is dissolved in carbon tetrachloride than in chloroform.

In procedures previously described, an excess of dithizone was added to insure the combination of all the zinc with dye. The excess dithizone was removed from the extracted zinc dithizonate by washing with dilute ammonia. This step, however, may introduce a considerable error because it is extremely difficult to determine the end-point of the washing process. In the technique described, the excess dithizone is not removed. The amount of zinc dithizonate present is determined by colorimetry at two critical wave-lengths, as described below.

Reagents—All reagents must be absolutely zinc-free. The best grade of chemically pure reagents should be obtained and, in our experience, even these may contain zinc in amounts sufficient to result in significant errors in the range of zinc content in which we are interested. Reagents therefore may require purification, as described below.

1. Diphenylthiocarbazone (Eastman Kodak). 100 mg. are dissolved in 1000 cc. of carbon tetrachloride, c. p. This stock solution is diluted to 1 mg. per cent for the extraction of very small quantities of zinc (1 to 20 γ). This solution should be stored at 4-6° and protected from sunlight at all times. No observable change in the solution occurs on standing at room temperature for a few hours.

2. Buffer solution. 556 gm. of Na₂S₂O₃, c. p., 90 gm. of C₂COONa, c. p., and 10 gm. of KCN, c. p., are dissolved in 1000 cc. of zinc-free water. The solution is then titrated with 15 N CH₃COOH to an approximate pH of 5.5, with methyl red as an indicator. A final adjustment to pH 5.5 is then made with a sensitive pH meter. The solution is then made up to 2000 cc. with zinc-free water in a volumetric flask. The buffer is then shaken with dithizone in carbon tetrachloride to remove any contaminating zinc, the extraction being repeated in a 500 cc. separatory funnel until the dithizone remains a clear green.

3. Tartrate solution. A 20 per cent solution of NaKC₇H₄O₆·4H₂O,
c.P., is made up with zinc-free water. The solution is extracted with dithizone as in (2).

4. 0.1 N NH₄OH, c.P.
5. Concentrated NH₄OH, c.P.
6. Methyl red indicator, 1:100 alcoholic solution.

Cleaning of Glassware—Pyrex glassware must be used throughout the procedure since ordinary soft glass contains zinc. Particular precautions must be observed to prevent contamination with zinc. All glassware, including separatory funnels, transfer funnels, volumetric flasks, beakers, transfer and capillary pipettes, colorimetric tubes, test-tubes, and syringes for drawing blood, is washed with soap and water, rinsed with single distilled H₂O, and then immersed in a bath of 2 N HNO₃ for a minimum of 6 hours, preferably overnight. On removal from the acid, the glassware is rinsed with zinc-free water, to which a few drops of methyl red have been added, until disappearance of the indicator's red color demonstrates the removal of all acid. Satisfactory zinc-free water may be obtained by double distillation, the second of which is done in an all-Pyrex glass still. Previous to use, separatory funnels are then shaken (several times if necessary) with about 20 cc. of buffer solution and about 5 cc. of 1 mg. per cent dithizone solution, until the dithizone in the funnel remains green. The dithizone is discarded. An identical procedure is employed for cleaning volumetric flasks. In addition, flasks are then rinsed with 0.01 N NH₄OH until yellow to methyl red. This insures that the pH of any residual rinsing water will be within the range of alkalinity at which the extracted zinc dithizonate is not affected.

For routine dry ashing of blood samples which contain only a total of from 1 to 20 γ of zinc, platinum crucibles must be used. In this range porcelain, Vicor, Pyrex, and quartz crucibles have been found unsatisfactory, since apparently all contain minute quantities of zinc. They may be used safely for tissue samples, provided the aliquots are large enough so that the error introduced is negligible. Both platinum and porcelain crucibles are cleaned by boiling 2 N HCl in the vessel for at least 30 minutes, after which they are rinsed several times with zinc-free water.

Method

We have routinely analyzed whole blood, erythrocyte, leucocyte, and plasma fractions thereof (9), and samples of internal organs, bone, and urine. The material is placed in the crucible and slowly evaporated on a hot-plate until almost dry. The crucible is then placed in an electric oven, at room temperature, and the furnace temperature raised to 600°. Complete ashing requires from 12 to 24 hours.

The ash is boiled in the crucible on a hot-plate with 15 to 30 cc. of 2
n HCl until completely dissolved, the amount of acid used depending on
the quantity of ash. The dissolved ash is evaporated to a volume of
about 5 cc. and is then transferred to a 125 cc. Squibb separatory funnel
by repeated washing with small portions of hot zinc-free water. When
analyzing tissue samples containing more zinc than blood samples, it is
desirable to bring the acid-ash solution up to 25 or 50 cc. in a volumetric
flask and extract a fraction thereof.

2 cc. of the tartrate solution, together with 2 drops of methyl red, are
added to the acid-ash solution in the separatory funnel. Stop-cocks are
geared with silicon. The contents of the funnel are then titrated with
NH₄OH and H₂SO₄ to pH 5.5, at which methyl red has a peach color.
50 cc. of buffer are added and the contents are allowed to stand until the
color has completely faded. Dithizone in CCl₄ (about 10 cc. of a 1 or 10
mg. per cent solution, depending on the amount of zinc present) is added,
and the funnel is shaken vigorously for about 2 minutes. The dithizone
in CCl₄ solution is allowed to collect in the bottom of the funnel, the last
drop is shaken down, and the CCl₄ phase is drawn off into a 50 cc. volu-
metric flask. This procedure is repeated until the dithizone in the funnel
remains a clear green. The sample in the volumetric flask is brought to
volume with CCl₄. Depending upon the quantity of excess dithizone
present, the final color may be purple or have a greenish tinge.

Colorimetry.—Dithizone in CCl₄ has an absorption maximum at 620 mμ;
zinc dithizonate has an absorption maximum at 520 mμ, but is transparent
at 620 mμ. With the filters employed, the ratio of the relative optical
densities of dithizone at 620 and 520 mμ has a numerical value of 4.65
(see Fig. 1).

Readings are obtained on extracted samples at both 520 and 620 mμ
with the Evelyn macro photocolorimeter (10). Since the galvanometer
used has an optimal accuracy within the range of from 40 to 80 per cent
transmission, the final dilution is made with CCl₄ to insure readings falling
within this region.

The zinc content of samples is calculated from the equation

\[
Z = \frac{L_{520} - L_{620}}{R} \times D \times K \times 100
\]

in which \(Z\) = total zinc in micrograms in the entire sample; \(L_{520}\) = density
(2 - log of the galvanometer reading) at 520 mμ; \(L_{620}\) = density at 620 mμ;
\(R\) = the ratio of density of dithizone in CCl₄ at 620 mμ and 520 mμ (nu-
merical value determined as 4.65, see Table I); \(D\) = the dilution factor
with relation to the original volume (\(V\)); \(K\) = the calibration constant
(numerical value 40; see Table II); \( V \) = the volume in which all of the extracted zinc dithizonate is originally diluted.

The ratio of absorption of dithizone at 620 and 520 m\( \mu \) (\( R \) in equation (1)) was determined as follows. A solution of dithizone in CCl\(_4\) was prepared of such a concentration as to deflect the galvanometer to about 10 per cent of the full scale at 620 m\( \mu \). A series of dilutions was then made from this so that in the lowest concentration the galvanometer registered about 80 per cent transmission. The series was then read at both wave-

![Absorption curves of dithizone and zinc dithizonate in carbon tetrachloride.](image)

**Fig. 1.** Absorption curves of dithizone and zinc dithizonate in carbon tetrachloride.

lengths. The data are given in Table I. In every instance the observed value at 620 m\( \mu \) was within 2 per cent of the value to be expected from the original concentration and dilution factor. Values for \( R \) averaged 4.65 \( \pm \) 1 per cent.

**Calibration**—A stock solution of zinc chloride was prepared by dissolving 10 mg. of metallic zinc in concentrated HCl and making up to a final volume of 1000 cc. From this solution a series of standards containing from 4 to 50 \( \gamma \) was prepared. These standards were extracted as described
above, except that they were not dry ashed. The total zinc dithizonate was made up initially to 50 cc. Further dilutions with CC\textsubscript{4} were then made to obtain galvanometer readings within the optimal range of the instrument. The data obtained are given in Table II.

**Table I**

*Ratio of Absorption of Dithizone at 620 \textmu m and 520 \textmu m*

<table>
<thead>
<tr>
<th>Dilution factor</th>
<th>Galvanometer reading</th>
<th>(L_{620})</th>
<th>(L_{520})</th>
<th>(R = \frac{L_{620}}{L_{520}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(0)</td>
<td>10.25</td>
<td>0.989</td>
<td>0.999</td>
<td>4.63</td>
</tr>
<tr>
<td>(0.50)</td>
<td>31.75</td>
<td>0.498</td>
<td>0.072</td>
<td>4.67</td>
</tr>
<tr>
<td>(0.33)</td>
<td>47.00</td>
<td>0.330</td>
<td>0.072</td>
<td>4.60</td>
</tr>
<tr>
<td>(0.33)</td>
<td>46.00</td>
<td>0.337</td>
<td>+2.1</td>
<td>4.70</td>
</tr>
<tr>
<td>(0.20)</td>
<td>63.75</td>
<td>0.196</td>
<td>0.043</td>
<td>4.63</td>
</tr>
<tr>
<td>(0.16)</td>
<td>69.50</td>
<td>0.158</td>
<td>0.034</td>
<td>4.63</td>
</tr>
<tr>
<td>(0.10)</td>
<td>79.25</td>
<td>0.101</td>
<td>0.021</td>
<td>4.70</td>
</tr>
</tbody>
</table>

**Average........

4.65 ± 0.04

**Table II**

*Calibration Constant for Zinc Dithizonate-Dithizone Solutions in CC\textsubscript{4}*

<table>
<thead>
<tr>
<th>Total Zn</th>
<th>Dilution factor</th>
<th>(L_{620})</th>
<th>(L_{520})</th>
<th>(L_{620}^{4.65})</th>
<th>Corrected (L_{520})</th>
<th>(K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>0</td>
<td>0.254</td>
<td>0.305</td>
<td>0.066</td>
<td>0.198</td>
<td>40.2</td>
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<tr>
<td>10</td>
<td>2</td>
<td>0.321</td>
<td>0.385</td>
<td>0.083</td>
<td>0.238</td>
<td>42.1</td>
</tr>
<tr>
<td>20</td>
<td>3</td>
<td>0.382</td>
<td>0.274</td>
<td>0.088</td>
<td>0.324</td>
<td>41.1</td>
</tr>
<tr>
<td>30</td>
<td>4</td>
<td>0.453</td>
<td>0.280</td>
<td>0.062</td>
<td>0.393</td>
<td>38.2</td>
</tr>
<tr>
<td>40</td>
<td>5</td>
<td>0.462</td>
<td>0.242</td>
<td>0.052</td>
<td>0.410</td>
<td>39.0</td>
</tr>
<tr>
<td>50</td>
<td>5</td>
<td>0.553</td>
<td>0.211</td>
<td>0.045</td>
<td>0.508</td>
<td>39.5</td>
</tr>
</tbody>
</table>

**Average........

40.0 ± 2.0

The value for \(K\) was calculated from equation (2), derived from equation (1).

\[
(2) \quad K = \frac{2Z}{\text{Corrected } L_{620} \times D}
\]

The numerical value of \(K\) averaged 40.0 ± 2.0, or ±5 per cent.

Inasmuch as this modification of the dithizone method was designed for the analysis of the zinc content of blood samples, it was necessary to deter-
mine to what extent the accuracy of the measurement was affected by the dry ashing process.

A stock solution of the ZnCl₂ was made up to contain approximately 1 γ of Zn per cc. A series of samples, in duplicate, was prepared containing total quantities of 2, 5, 10, 15, 20, and 30 γ of zinc. These were extracted without dry ashing. The results are given in Table III. The average value of Zn in micrograms per cc. was 0.95, with a standard deviation of 0.05. This was taken as the correct concentration of the stock solution.

An identical series was then dry ashed and extracted, the values obtained also being given in Table III. An average value of 0.97 γ of Zn

| Table III |
| Standardization of Zinc in Samples of Blood Components |

<table>
<thead>
<tr>
<th>Amount analyzed</th>
<th>Stock solution</th>
<th>Stock solution, dry ashed</th>
<th>Dry ashed with white cells, 0.572γ</th>
<th>Dry ashed with red cells, 3.69γ</th>
<th>Dry ashed with plasma, 4.29γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>cc.</td>
<td>total γ</td>
<td>γ per cc.</td>
<td>total γ</td>
<td>γ per cc.</td>
<td>total γ</td>
</tr>
<tr>
<td>2</td>
<td>1.63</td>
<td>0.81</td>
<td>1.79</td>
<td>0.89</td>
<td>2.75</td>
</tr>
<tr>
<td>2</td>
<td>1.76</td>
<td>0.88</td>
<td>1.77</td>
<td>0.88</td>
<td>2.31</td>
</tr>
<tr>
<td>5</td>
<td>4.80</td>
<td>0.97</td>
<td>5.14</td>
<td>1.00</td>
<td>5.46</td>
</tr>
<tr>
<td>5</td>
<td>4.81</td>
<td>0.96</td>
<td>5.08</td>
<td>0.99</td>
<td>4.92</td>
</tr>
<tr>
<td>10</td>
<td>9.9</td>
<td>0.99</td>
<td>9.99</td>
<td>0.82</td>
<td>10.57</td>
</tr>
<tr>
<td>10</td>
<td>9.88</td>
<td>0.98</td>
<td>8.20</td>
<td>1.06</td>
<td>12.59</td>
</tr>
<tr>
<td>15</td>
<td>14.80</td>
<td>0.88</td>
<td>16.14</td>
<td>1.05</td>
<td>17.48</td>
</tr>
<tr>
<td>15</td>
<td>14.36</td>
<td>0.96</td>
<td>15.52</td>
<td>1.00</td>
<td>18.08</td>
</tr>
<tr>
<td>20</td>
<td>19.27</td>
<td>0.96</td>
<td>20.07</td>
<td>0.99</td>
<td>19.66</td>
</tr>
<tr>
<td>20</td>
<td>19.00</td>
<td>0.95</td>
<td>19.83</td>
<td>0.99</td>
<td>19.66</td>
</tr>
<tr>
<td>30</td>
<td>29.18</td>
<td>0.97</td>
<td>29.88</td>
<td>0.99</td>
<td>31.36</td>
</tr>
</tbody>
</table>

Mean*... 0.95 0.97 0.96 0.98 0.93
s.d., σ... ±0.05 ±0.069 ±0.08 ±0.112 ±0.155

* Over-all mean = 0.96; over-all s.d., σ = ±0.0985.

per cc., with a standard deviation of 0.07, was obtained, indicating that there was no loss of the metal in the ashing. It will be noted that in both series the values for the 2 γ standards were low by about 10 per cent, averaging 0.86 γ per cc. This apparent loss probably is due to slight errors in colorimetry, since similar low values were not obtained in the series described below.

Three additional series were processed, in which known amounts of zinc (2 to 30 γ) were added to samples of separated canine white cells, red cells, and of plasma. In each series the cells and plasma were obtained by floatation of 10 cc. aliquots of the same blood. Individual floatations
were done for each standard, and hence the amount of zinc due to cells and plasma was the same at all concentrations of zinc. The total amount of zinc in the red and white cells and plasma was determined, in duplicate, and the average value subtracted from the total zinc found in the several standards, to obtain the net amount of zinc recovered. Data are given in Table III. The average net zinc recovered (in micrograms per cc.) and standard deviation was 0.96 ± 0.08, 0.98 ± 0.112, and 0.93 ± 0.155 μg, in the white cell, red cell, and plasma series, respectively. These values compare well with those found for the series that did not contain cells or plasma.

These experiments constitute a check on the over-all accuracy of the procedure, from the separation of cells and plasma from whole blood to the final colorimetric measurement. It would appear that the limit of error lies within ±5 per cent.

As a check on the absence of contamination of all glassware with extraneous zinc, we have found it desirable to determine a 5 or 10 μg standard with each day's set of extractions. This extraction is carried out with a random selection of crucibles, separatory funnels, volumetric flasks, pipettes, etc. Results obtained in fifteen consecutive analyses of such standards show a mean value of 9.82 μg, with a standard deviation of ±0.65. The narrow limits of error found reflect the degree of prevention of contamination in all stages of the procedure.

Since our investigation of zinc metabolism involves the use of the radioactive isotope of zinc, Zn⁶⁸, it is desirable that measurements of both total and radioactive zinc be made on the same blood or tissue sample. To effect the conversion of zinc dithizonate to a water-soluble zinc salt, all of the extracted zinc dithizonate is returned to a clean separatory funnel, a drop of concentrated H₂SO₄ is added, followed by 10 cc. of water, and the funnel is shaken until all the zinc has gone into the aqueous phase, as evidenced by the return of the carbon tetrachloride phase to the green color of dithizone. We have found this method satisfactory in the analysis of blood and tissue samples in dogs (11), in normal humans (12), and in the leucemias, blood dyscrasias, and various other pathological conditions.

SUMMARY

A modification of the dithizone method of extracting zinc from blood and tissue samples is described. The procedure permits of accurate assay of total zinc content of samples in amounts as small as 1 μg.

We wish to acknowledge the technical assistance of Miss Mary L. Roney.

1 Gibson, J. G., 2nd, and Vallee, B. L., in preparation.
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