Determination of Calcium in Urine by the Chloranilate Method

William W. Webster and William H. Kern

Evidence is presented that the colorimetric chloranilate calcium method can be accurately applied to urine provided that elimination of interfering substances, which include magnesium, is performed prior to the determination. Isolation of calcium as the oxalate, followed by perchloric acid digestion, has been shown to be an acceptable method whereby urinary calcium can be put in a suitable state for analysis. Allowing the urine to stand for 1 hr. at pH 2 prior to the oxalate precipitation has been shown to be a simple means of eliminating the sampling error due to calcium precipitates.

The use of chloranilic acid in the colorimetric estimation of calcium, as originally reported (1, 2) and later modified in our laboratory (3), has proven to be a reliable procedure when applied to serum (4). However, the direct application of serum calcium methods to urine has been complicated by the problem of insoluble calcium precipitates and the presence of interfering substances in the urine. Thus Fales (5) has found that neither chelometric nor permanganate titration methods can be applied directly to urine without prior isolation of calcium to remove interfering substances. The precipitation of calcium oxalate, followed by oxidation with perchloric acid, was concluded to be a simple means to prepare the sample for analysis. In addition, Fales recommended boiling the urine with nitric acid prior to analysis in order to dissolve insoluble calcium precipitates.

The direct application of the chloranilate method to urine has been proposed in the Lab-Trol manual (6) and by Chiamori and Henry (7). The Lab-Trol procedure calls for bringing the urine sample to pH 5–7, filtering, and proceeding as for serum; no data were given regarding the problem of calcium precipitates nor the existence of possible interfering substances in the urine. Chiamori and Henry boiled the urine with

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acid to dissolve the calcium precipitates and compared the results of the
direct chloranilate procedure with parallel analyses on ashed specimens
determined by the Clark-Collip procedure (9). Those workers found
levels of calcium by the direct chloranilate procedure higher than by the
permanganate titration, but came to no conclusions as to the relative ac-
curacy of the two procedures.

The present investigation was undertaken to determine the magnitude
of possible error introduced by the direct application of the chloranilate
calcium procedure to urine. On the basis of our findings, as presented in
this communication, that the direct method exhibits inadequate specifi-
city, the following procedure incorporating isolation as the oxalate was
adopted for the determination of urinary calcium.

Reagents

**Oxalic acid, 2.5% (W/V)**

**Methyl orange indicator 1% (W/V)**

**Sodium acetate 20% (W/V)**

**Perchloric acid 30%** Dilute 43 ml. of 70% perchloric acid to 100 ml.
with distilled water.

**Chloranilic acid** (*Baker's Analyzed Reagent*) Dissolve 1 gm. of
chloranilic acid in approximately 50 ml. of distilled water containing 7
ml. of 1N NaOH. Mix and dilute to 100 ml. with distilled water. Filter
before use if crystallization occurs.

**Isopropyl alcohol, 50% (W/V)**

**Tetrasodium ethylenediaminetetraacetate, 5% aqueous (W/V)**

**Ferric chloride, approximately 6% aqueous** Weigh out approximately 10 gm. of FeCl₃·6 H₂O and dissolve in 100 ml. of distilled water.

**Ferric chloride, approximately 0.6% aqueous** Prepare daily by
means of mixing 1 part of 6% ferric chloride solution with 9 parts of
distilled water.

**Calcium standard (5 mEq./L.)** Weigh out 0.2497 gm. of reagent
grade calcium carbonate and transfer it to a 1-L. volumetric flask. Dis-
solve the salt in 10 ml. of 1N HCl and make to volume with distilled water.

Method

Bring the total 24-hr. urine to pH 2 with concentrated HCl using wide-
range pH paper, mix well, and allow to stand 1 hr. Mix the urine again
and measure the total volume. Place 5 ml. of acidified urine in a 15-ml.
conical centrifuge tube and add 1.0 ml. 2.5% (W/V) oxalic acid and 2
drops of 1% (W/V) methyl orange indicator. Bring the urine to a
yellow-orange end point (pH 4.2) with 20% sodium acetate and allow the
calcium to precipitate overnight. Centrifuge the tube at high speed for 10 min. Decant the supernatant fluid carefully and allow the tube to drain.

Dissolve the precipitate in 0.5 ml. of concentrated nitric acid, add 2 ml. of water, and transfer the contents to a small porcelain casserole. Rinse the tube with 1 ml. of 30% perchloric acid and 1 ml. of water, transferring both washes quantitatively to the casserole.

Evaporate the mixture on low heat on a hot plate, taking approximately 45 minutes to complete digestion. Take care to maintain the mixture just below boiling, with a slow steady evolution of fumes towards the end, since perchlorates are involved. When this is cool, add 4.0 ml. of water to dissolve the ash, and bring the solution to pH 6–7 with 1N NaOH.

Transfer the solution to a 15-ml., graduated centrifuge tube and adjust the volume to 5.0 ml. with water; mix.

Into conical 15-ml. centrifuge tubes pipette as follows.

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Digested sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>0.5 ml.</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Standard</td>
<td>—</td>
<td>0.5 ml.</td>
<td>—</td>
</tr>
<tr>
<td>Sample</td>
<td>—</td>
<td>—</td>
<td>0.5 ml.</td>
</tr>
<tr>
<td>Chloranilic acid</td>
<td>0.25 ml.</td>
<td>0.25 ml.</td>
<td>0.25 ml.</td>
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</table>

Mix and allow to stand 60 min., minimum time. (The 30-min. period previously specified for serum calciums has been found inadequate for certain specimens.)

Centrifuge at high speed 10 min., decant the supernatant fluid, and drain. Wash with approximately 5 ml. of 50% isopropyl alcohol, centrifuge, and drain.

Add 2 drops of 5% EDTA and dissolve the precipitate. (A Vortex mixer* or Cyclomixer † is very helpful at this stage.) By means of a calibrated syringe, add 5.0 ml. of 0.6% ferric chloride. Mix well and transfer to cuvets.

Read the solutions on a colorimeter at 490 m\(\mu\) or on a Klett instrument with No. 54 filter. The color is stable.

Calculation is as follows.

\[
\text{Klett units (or O.D.) of unknown} \times 5.0 = \text{mEq. Cal/L. of urine}
\]

**Experimental**

The method as finally evolved and detailed above is essentially a combination of the procedure which Fales (5) employed for isolation and

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*Kraft Manufacturing Company, New York, N. Y.
†Clay Adams Inc., New York, N. Y.
digestion of the calcium oxalate and the method for serum calcium as previously reported from our laboratory (3). Fales, however, employed a rather more laborious method of dissolving the calcium precipitates by boiling 25 parts of urine with 1 part nitric acid for 3 min. We therefore decided to test the necessity of the boiling procedure. Urine samples were brought to pH 2 (using wide range pH paper) with concentrated HCl mixed well, allowed to stand 1 hr., and mixed again. Then 1 ml. of concentrated nitric acid was added to 25 ml. of urine and boiled 5 min. After cooling, the urine was brought to pH 6-7 with 5N NaOH and the volume noted for adjusting the final calculation. In addition, unboiled urine at pH 2 was brought to pH 6-7 with 5N NaOH, and 0.5-ml. aliquots analyzed. Twenty-six urines were analyzed in duplicate directly on the boiled and unboiled urines. Comparison of the calcium levels shown in Fig. 1 demonstrates that the two methods of dissolving the calcium precipitates yield essentially the same results. The calcium level of the unboiled urines averaged 0.07 mEq./L. higher than that of the boiled urines, but the difference was not significant (0.2<P<0.3) by the t test (8). It was concluded that standing at 1 hr. at pH 2 is sufficient to dissolve the precipitates and, therefore, the boiling with concentrated nitric acid was omitted as a routine procedure.

We next tested the necessity of isolating the calcium as the oxalate.

Twenty-nine urine samples were analyzed in duplicate directly on urine and compared with oxalate digests. The average calcium level of the urines analyzed directly was 0.63 mEq./L. higher than the average level obtained on the digests. This difference was highly significant (P<.001).
and compares well with the results of a similar study by Chiamori and Henry (7), who found that the direct chloranilate procedure yielded average values 1.3 mg./100 ml. (or 0.65 mEq./L.) higher than the level obtained by the Clark-Collip procedure applied to ashed specimens. If this difference were constant it might be unimportant for all practical purposes; however, it is quite variable, ranging from no detectable difference in some urines up to a 30% increase in others when the chloranilate reaction is applied directly to urine. This variability is demonstrated in Fig. 2. Since Chiamori and Henry brought the urine to a pH of 4.5 before adding the chloranilate, we compared 8 urines precipitated at this pH against the same urines brought to pH 6–7; there was no statistical difference, 0.3<P<0.4, between the results of the two methods of precipitation.

The present method was compared with the Clark-Collip procedure on aliquots of digested oxalate precipitates of 12 different urines. The correlation between the two methods is shown in Fig. 3. The chloranilate method yielded results averaging 0.08 mEq./L. higher than the Clark-Collip with no significant difference (0.3<P<0.4) between the two methods. To test the precision of the proposed method, 11 aliquots of the same urine were precipitated as the oxalate and digested, and duplicate analyses were performed by the chloranilate procedure; at an average level of 12.3 mEq. the standard deviation was 0.25 mEq./L. This compares with a previously reported (3) standard deviation of 0.14 mEq./L. at a level of 4.45 mEq./L. calcium by the same method applied directly to replicate samples of serum.

![Fig. 2](image-url) Urine calcium values obtained by chloranilate method applied directly to urine and to calcium oxalate digests.
No systematic attempt was made to identify all of the substances which might interfere with the direct application of the chloranilate reaction to urine. Magnesium, which has been reported not to interfere in the determination of serum calcium (1), was investigated since the molarity of urinary magnesium may exceed that of calcium by 800% (5), whereas its concentration in serum seldom exceeds that of calcium. When a mixture of 2.5 mEq./L. calcium and 2.5 mEq./L. magnesium (representing an extremely low serum calcium and elevated serum magnesium) was analyzed, no interference was observed. However, when a mixture of 2.5 mEq./L. calcium and 40.0 mEq./L. magnesium was analyzed, a spurious elevation of approximately 1 mEq./L. calcium was observed (average of four replicate determinations). Thus, at least part of the interference in the application of the chloranilate calcium method directly to urine may be attributed to the relatively greater concentration of magnesium in urine than in serum. Since oxalate precipitation of the calcium at pH 4.2 allows magnesium to remain in solution (5), the present method would eliminate this potential source of error.

References


