Determination of Calcium in Biologic Material by the Chloranilate Method. (23888)

NEIL CHIAMORI AND RICHARD J. HENRY
Bio-Science Research Foundation, Los Angeles, Calif.

Calcium is precipitated by chloranilic acid, a reagent which is red-purple. Teeri(1) applied this reaction in determining calcium in serum, first separating calcium as the oxalate, then dissolving the oxalate with acid and determining calcium by photometrically measuring decrease in color of chloranilate added in excess. Ferro and Ham(2,3) approached the analysis in a different way, precipitating calcium from serum as chloranilate, then dissolving the latter in a solution of ethylenediamine tetraacetate at high pH and determining color of liberated chloranilate photometrically. In comparing the method of Ferro and Ham with that of Clark and Collip(4), the present authors experienced difficulty with the former method because of turbidity in the final colored solution in a significant number of samples. This communication deals with a solution to this problem, applies the method to determination of calcium in urine and feces and gives comparative results obtained by chloranilate and Clark-Collip methods. An ultramicro adaptation of the method to serum is also presented.

Methods. Reagents. 1. Chloranilic acid reagent. Dissolve 4 g of NaOH pellets in about 650 ml distilled water in a liter volumetric flask. Add 11 g of chloranilic acid (E.K. No. P 4539) and make up to 1 liter with water. Agitate until acid dissolves. The pH should be less than 11. Usually the pH is about 7 but, if greater than 11, additional 1 g quantities of chloranilic acid are added to reduce the pH. Filter through a Whatman No. 1 paper using a Buchner funnel. Filter again through a Seitz pad, No. ST-1 or ST-3 (Hercules Filter Corp., Size L-6). The reagent is stable in refrigerator for about 6 months but only 2 to 3 months at room temperature(3). 2. Tetrasodium ethylenediamine tetraacetate, 5% (EDTA reagent). Ferro and Ham employed tetrasodium salt but, since this is difficult to obtain, the required reagent can be prepared from disodium ethylenediamine tetraacetate dihydrate (Baker, A. R. grade) as follows: Add 44.8 g of disodium salt to about 800 ml of water in a liter volumetric flask, dissolve the salt by adding 10% NaOH drop by drop until pH 10.3 is reached, then add water to 1 liter. 3. Isopropyl alcohol, 50% (V/V). 4. Calcium standard, 200 μg/ml. Weigh exactly 500 mg Iceland Spar and transfer to a 1-liter volumetric flask. Add 14 ml dilute HCl (1 part conc acid + 9 parts distilled water). Let stand until solid is dissolved, then add distilled water to 1 liter. *Ultramicro procedure for serum.* Set up the following in 2-ml conical-tipped centrifuge tubes: *Blank.* 100 μl water. *Standard.* 100 μl calcium standard containing 20 μg of calcium. *Serum.* 100 μl of serum. To each tube add 100 μl of chloranilic acid reagent, mix and let stand 2 hours. (Contrary to the claim(3) that 30 minutes are sufficient for complete precipitation of calcium chloranilate from serum samples, the present authors found that 2 hours were required as a safe minimum.) Centrifuge and remove supernate either by carefully decanting or by siphoning with reversed tipped capillary. Invert and drain 3 minutes, then wipe mouths of tubes with absorbent tissue. Wash precipitate with 0.15 ml of 50% isopropyl alcohol, stirring precipitate with a rod. Wash rod with 0.1 ml wash reagent. Centrifuge, discard supernate, invert and drain. Repeat washing procedure. Dissolve washed precipitate in 0.3 ml EDTA reagent, stirring with glass rod. Centrifuge. Examine unknown for turbidity by checking for Tyndall effect. If turbid, extract with 0.15 ml ethyl ether. Centrifuge and read absorbances of clear, aqueous phases against distilled water at 525 mμ, the absorbance peak, in Bessey-Lowry micro cuvets (Pyrocell Mfg. Co., N. Y. 28) in Beckman Model DU spectrophotometer. Occasionally, in an extremely lipemic serum, it may be necessary to extract with 2 aliquots of

ether for clarity. Calculation: $\frac{A_X - A_B}{A_S - A_B} \times 20 =$ mg Ca/100 ml serum. *Macro procedure for serum.* Similar to ultramicro procedure except that 2 ml quantities of distilled water, standard and unknown are placed in 12-ml, heavy wall centrifuge tubes and 1 ml chloranilic acid reagent added. Precipitates are washed with 5 ml isopropyl alcohol and finally dissolved in 6 ml EDTA reagent. If turbid, extract with about 3 ml of diethyl ether. Absorbances can be read in a spectrophotometer at 525 mμ or with a filter with nominal wavelength in this region (Beer's law was followed at 525 mμ on Beckman Model DU spectrophotometer and on a Klett photometer with a No. 54 filter). Calculations are the same as for ultramicro procedure. *Procedure for urine.* Transfer 10.0 ml of well-mixed urine to a test tube. Acidify to at least pH 1 by adding several drops of conc. HCl and testing with pH paper. Mix, mark meniscus on the tube and place in boiling water for about 30 minutes. Cool to room temperature, add water to mark, mix and centrifuge. Pipet 2.0 ml of supernate to a 12-ml, thick-walled centrifuge tube, adjust pH to 4.5 with 2% NH_4OH using narrow range pH paper, add 1.0 ml chloranilic acid reagent and proceed and calculate as for serum. *Procedure for feces.* Weigh approximately 1 g of feces into a small porcelain crucible. Dry sample on a steam bath. Ash with gentle flame for a few minutes until most of the carbon is burned off. Cool and add a few drops of water. Mix and break up the remaining carbon with a stirring rod. Dry on the steam bath. Ash again until no carbon remains. Cool and add 3 drops of 6 N HCl to dissolve the ash. Dry again on the steam bath. Add 2 more drops of 6 N HCl and transfer quantitatively with hot distilled water into a 12-ml, thick-walled, graduated centrifuge tube. Add water to 10.0 ml and mix. Transfer 1.0 ml to another centrifuge tube, add 1 ml of water, adjust the pH to 4.5 with 2% NH_4OH using narrow range pH paper. Add 1.0 ml chloranilic acid reagent and proceed as for serum. Calculation:

$$\frac{A_X - A_B}{A_S - A_B} \times \text{mg Ca in standard} \times \frac{1000}{\text{g wet weight of feces in sample}} =$$

mg Ca/100 g wet weight of feces. *Clark-Collip method.* The method of Clark and Collip(4) was followed for serum except that the calcium oxalate was quantitated by perchloratoceric acid titration using ferroin as indicator(5). For urine and feces the method was applied to ashed specimens.

TABLE I. Interference by Turbidity.

Clark-Collip, mg Ca/100 ml	Chloranilate: Unextracted, mg Ca/100 ml	Chloranilate: Unextracted, Turbidity	Chloranilate: Extracted, mg Ca/100 ml
9.0	9.1	0	9.1
10.1	9.6	0	9.6
9.1	9.4	1+	9.2
9.4	10.0	2+	9.5
9.5	12.4	3+	9.6
9.9	13.2	3+	9.7
9.1	11.6	4+	9.0

Results. Interference in analysis of serum by turbidity. Table I gives representative comparisons between results (means of duplicate analyses) obtained by the Clark-Collip method and the chloranilate method, with and without ether extraction. That the turbidity is lipid in nature is indicated by the facts that it was removed by ether as well as other lipid solvents and that, in general, the degree of turbidity increased with the lipid content of the sample (*e.g.*, samples 5-7 in Table I were grossly lipemic). The appearance of turbidity at the final stage could be reduced but not completely avoided by multiple washings of the calcium chloranilate precipitate with the isopropyl alcohol wash reagent. Because of the volume change of the aqueous phase when saturated with ether, the absorbance of standards and unknowns decreases with ether extraction. The mean decrease in absorbance of standards and clear unknowns was 5%. Thus, rather than reading extracted unknowns against extracted blanks and standards, a correction can be made. It is seen from Table I that gross positive errors can occur if interference by turbidity is not avoided. Turbidity was never observed in this study with urine or fecal samples.

Comparison of results with the method of Clark and Collip. Nineteen samples of serum were analyzed in duplicate by both methods. The results by the chloranilate method averaged 0.1 mg/100 ml greater than those ob-

tained by the Clark-Collip procedure but this bias was not significant at $p = 0.05$ by the t test(6). In a similar study of 21 urines, results by the chloranilate method averaged 1.3 mg/100 ml greater than those yielded by the Clark-Collip procedure. This difference was significant at $p = 0.05$ but not at $p = 0.01$ (t test). Similarly, results of fecal calcium were about 5 to 10% higher by the chloranilate method. The cause for these discrepancies is not apparent and it is impossible at this time to tell which method is more accurate. For most purposes, however, this difference is unimportant.

Reproducibility. The standard deviations (mg/100 ml), calculated from duplicate analyses, were as follows: 0.16 and 0.59 for the Clark-Collip method applied to serum and urine, respectively; 0.17 and 0.16 for the chloranilate method applied to serum and urine, respectively. This means that by the chloranilate method the precision (95% limits) of a single analysis of serum calcium was about $\pm$ 3.2% and that for a single analysis of urine calcium at a level of 25 mg/100 ml was about $\pm$ 1.5%.

Discussion. It is the authors' opinion that the chief advantage of the chloranilate method for the determination of serum calcium is the working time saved when many samples are to be run. The advantage of direct application to urine without preliminary ashing is obvious. The precision of the technic for both serum and urine is quite good and the results, in the case of serum, appear identical to those obtained by the Clark-Collip procedure. With urine and fecal samples, the results by the chloranilate method are slightly higher than those obtained by the Clark-Collip procedure.

Summary. The Ferro-Ham chloranilate procedure for determination of serum calcium has been investigated. Modifications of this procedure are presented for determination of urine and fecal calcium and ultramicro modification for determination of serum calcium is described. Presence of turbidity, apparently lipid in nature, in final solution constitutes a source of significant positive error in the chloranilate method for determination of calcium in serum. This turbidity can be removed by ether extraction. Results obtained by the chloranilate method have been compared with the Clark-Collip procedure.

1. Teeri, A. E., *Chemist Analyst,* 1954, v43, 18.
2. Ferro, P. V., Ham, A. B., *Am. J. Clin. Path.,* 1957, v28, 208.
3. ———, *ibid.,* 1957, v28, 689.
4. Clark, E. P., Collip, J. B., *J. Biol. Chem.,* 1925, v63, 461.
5. Ellis, G. H., *Anal. Chem.,* 1938, v10, 112.
6. Bennett, C. A., Franklin, N. L., *Statistical Analysis in Chemistry and the Chemical Industry,* John Wiley & Sons, N. Y., p180, 1954.

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Effect of Some Hormones on Calcium Balance in Elderly Subjects.* (23889)

G. TORO, P. G. ACKERMANN AND W. B. KOUNTZ
St. Louis Chronic Hospital and Division of Gerontology, Washington University School of Medicine, St. Louis, Mo.

Our earlier studies on calcium and phosphorus balance in elderly subjects have shown that calcium requirements are higher for such subjects than other workers found for young adults, with little change in phosphorus requirements(1,2). We later noted that addition of Vit. D to a supposedly adequate diet increased calcium retention in these subjects (3). Increased calcium requirements in the aged make it more likely that such subjects will be in negative calcium balance on an ordinary diet. The increase in incidence of osteoporosis in the aged has been noted by many workers(4). We have been studying the ef-

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