Improvements on the Automatic Determination of Micro Amounts of Serum Calcium

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Recent work has shown that calcium in 0.2 ml of blood serum can be titrated automatically in less than 10 sec. with a reproducibility of 1–2%. It is not necessary to deproteinize the serum, so the total procedure requires less than 1 min. This new procedure is presented because it decreases both the quantity of serum and analysis time per determination to one-fifth or less those for the previously described procedure (1).

Reagents

All solutions are prepared with deionized water.

Standard calcium solution (0.00250 M) Dissolve 0.2502 gm. dry A.R. CaCO₃ and approximately 0.24 gm. MgSO₄·7H₂O in 50–55 ml. 0.1 HCl and make up to 1 L. with water.

EDTA solution Dissolve 1.875 gm. of disodium salt of ethylenediaminetetraacetic acid (EDTA) in water and make up to 1 L. with water. Standardize this solution against the standard calcium solution and store in a polyethylene bottle.

Calcon solution Dissolve 0.020 gm. Calcon (Cl 202, Baker) in 25 ml. of methanol.

Sodium hydroxide, 0.3 N aqueous solution Stored in a polyethylene bottle.

Potassium cyanide, 2% (w/v) aqueous solution

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Automatic Titrator

The titrator* used was the same as previously (1) except for the following. A plastic holder for an 18-mm. o.d. test-tube titration cell was made as shown in Fig. 1, and mounted on the beaker platform.

![Fig. 1. Holder for test-tube titration cell.](image)

Optimum focusing of the light was obtained by adjusting the position of the light source so that the center of the light beam passed through the test tube and fell on the detector lens mounted on the right side of the "spectro" compartment. A 6-mm. glass rod stirrer and the micro delivery tip were positioned as shown in Fig. 1. It is important that the stirrer be positioned 2-4 mm. from the bottom of the tube and the delivery tip next to and at the end of the stirrer. Also, a direct-reading, constant-rate, motor-driven buret† was used with the 10-ml. barrel, which provides a titrant delivery rate of 1 ml./min. The buret is plugged into the valve outlet on the back of the "spectro-electro" unit. Since only about 0.1 ml. of titrant is used for an average sample, 80-100 titrations can be performed without refilling the buret.

A red-cellophane cut-off filter is used in the auxiliary filter holder together with the usual 650-mu interference filter which is dialed into position.

Titrant Standardization

Pipet 0.200 ml. (V_{Ca}) of standard calcium solution into the tube, and add 2.0 ml. of 0.3 N NaOH and one drop of Calcon indicator. Push the start button on the control unit; this starts only the stirring because the buret drive is in "off" position. After 10-15 sec. of mixing, turn on the constant-rate buret. The titrant delivery is stopped automatically at the end point and the dial reading, $R$, is recorded. (The

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†Model 8-11120-1, E. H. Sargent Co.
volume of titrant delivered is $2R/1000$ ml.) Empty the reaction vessel by inserting a polyethylene aspirator tube and drawing out the contents. Follow with rinsing and withdrawal of rinse solution. Prior to the next titration reset the buret dial to zero and turn the drive switch to “off.”

Repeat the titration above using 0.100 ml. of standard calcium solution and record the dial reading $R_1$. The blank, $B$, of the titration and the molarity $M_T$ of EDTA titrant are calculated by equations (1) and (2), respectively:

$$2R_1 - R = B$$

$$M_T = \frac{V_Ca \cdot M_Ca}{V_T} = \frac{0.200 (0.0025)}{0.002 (R - B)} = \frac{0.250}{R - B}$$

where $V_T$ is actual volume used for titration of 0.2 ml. of standard calcium solution.

**Determination of Calcium in Serum**

**Procedure 1**

Follow the same procedure given for the standardization of EDTA solution but use 0.200 ml. of serum ($V_S$) instead of the standard calcium solution. Record the dial reading, $R_S$ and calculate the calcium as milligrams Ca per 100 ml. of serum, using Equation 3, or more simply by using Equation 3a.

$$V'_T M_T F_Ca \left( \frac{100}{V_S} \right) = 0.002 \left( R_S - B \right) M_T (40.08) \left( \frac{100}{0.200} \right) =$$

$$40.08 M_T \left( R_S - B \right) = \text{mg. Ca/100 ml. serum.} \quad (3)$$

If $M_T = 0.00500$ M, Equation 3 becomes

$$(R_S - B) \ (0.200) = \text{mg. Ca/100 ml. serum.} \quad (3a)$$

**Procedure 2**

If the phosphate content of the serum is abnormally high, titrate a mixture of 0.100 ml. serum and 0.100 ml. standard calcium solution according to the procedure given for the standardization of titrant, and record the dial reading $R'_S$. The volume of titrant equivalent to the calcium added to the serum is $0.00025/M_T$ ml. Calculate the serum calcium by Equation 4, or more simply by Equation 4a.

$$\left[ 0.002 \left( R'_S - B \right) - \frac{0.00025}{M_T} \right] M_T (40.08) \left( \frac{100}{0.100} \right) = \left[ 2(R'_S - B) M_T - 0.25 \right]$$

$$(40.08) = \text{mg. Ca/100 ml. serum} \quad (4)$$
If $M_r = 0.00500$ M, Equation 4 becomes

$$(R'_3 - B - 25) (0.400) \text{ mg. Ca/100 ml. serum.}$$

(4a)

Results and Discussion

A typical set of data for 11 serum control samples, from four manufacturers, is presented in Table 1. Results are reproducible within 1–2%. The average value determined for each sample was well within the manufacturer's permissible variation, which was given as ± 2% (Sample 9) to ± 5% (Samples 1–5). Recovery studies were made on the same samples to provide an additional check on the method; 0.100 ml. of standard calcium solution was added to 0.100 ml. of serum by means of a microliter syringe. The recovery of Ca varied from 97 to 103%, with an average of 100.8%. The same precision and recovery were obtained when the method was applied to a large number of human blood serum samples. The method was tested by 21 students as part of an experiment, and after 1 hr. of instruction about the procedure and instrument, they obtained results reproducible within 2%.

When several bottles of EDTA were used, the variation in concentration by direct weight was less than 0.5%, the titrant was 0.00500 M, and the simplified equations 3a and 4a were used for calculations. However, in one case, the assay for EDTA was less than 98%. Therefore, the standardization step is recommended to determine the purity of a bottle of EDTA. Subsequently the appropriate amounts of EDTA can be weighed to prepare a 0.00500 M EDTA solution so that equations 3a and 4a can be used for calculations.

With the special microholder and reaction vessel, it is necessary to

<table>
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<tr>
<th>Sample</th>
<th>Calcium (mg./100 ml. serum)</th>
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<tbody>
<tr>
<td></td>
<td>Reported</td>
</tr>
<tr>
<td>1</td>
<td>10.2</td>
</tr>
<tr>
<td>2</td>
<td>10.0</td>
</tr>
<tr>
<td>3</td>
<td>9.9</td>
</tr>
<tr>
<td>4</td>
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focus the light so that the center of the incident beam is centered on the solution and a sufficient amount of light falls on the detector. Differences in the focusing of the light and in the position of the stirrer and delivery tip might lead to differences in the value of blank. The blank can be determined precisely by titrating several 0.2-ml. aliquots and 0.1-ml. aliquots of the standard calcium solution. Over a period of several weeks the blank was 0.014 ± 0.001 ml.

When calcium solution containing no magnesium was used as standard, the blank was smaller by about 0.001 ml. as compared with serum samples or calcium solution containing small amounts of magnesium. It seems that the end point is retarded slightly in the presence of magnesium hydroxide. Therefore, magnesium is added to the standard calcium solution in amounts approximately equivalent to those found in an average blood serum sample to duplicate more closely the conditions present in serum samples.

When abnormally high concentrations of both calcium and phosphate are present, either premature end-points are obtained, or, sometimes, there is no automatic termination at the end point. This is caused by precipitation of calcium phosphate at the high pH (about 13) of the solution. In such cases only 0.100 ml. of serum is used for the titration. However, more accurate and precise results were obtained when 0.100 ml. of standard calcium solution was added to 0.100 ml. of serum (Procedure 2). Abnormal serum Samples 4 and 5 containing 8.2 and 8.0 mg. P-inorganic per 100 ml. serum, respectively, were analyzed by both procedures, and the results obtained agreed within ± 2%. Abnormal serum Samples 10 and 11 containing 12.4 and 7.1 mg. P per 100 ml., gave premature end points when Procedure 1 was used.

Addition of potassium cyanide solution to mask iron and copper was not necessary in the analysis of serum control samples and of several human serum samples. However, in hemolyzed serum, a drop of cyanide solution should be added and the solution stirred prior to the addition of Calcon indicator.

A red-cellophane filter is used instead of the yellow cut-off filter supplied with the instrument because it cuts out completely the radiation transmitted by the 650-mµ interference filter in the short wavelength region of the visible. However, the yellow cut-off filter can be used.

Reference