Evaluation of an Automatic Calcium Titrator

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An automatic calcium titrator for determining total serum calcium concentration has been evaluated. The instrument incorporates a motorized buret, a fluorometer, and a digital readout that is responsive to the quenching of the fluorescent calcium-calcein complex by the chelating agent, ethyleneglycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid. Analyses made with the calcium titrator were compared with those made with the SMA 12/60 and atomic absorption spectrophotometry. Good correlation was obtained in each instance. Slight hemolysis and bilirubin concentrations near normal did not affect the results; however, increased concentrations of these substances resulted in decreased values. The precision of analysis depends on the technique used in pipetting the sample. A single analysis of 0.1 ml of serum can be completed in 1 to 2 min. With careful analytical technique, precision is good (CV, 0.72%).

Additional Keyphrases  atomic absorption spectrophotometry • AutoAnalyzer • effect of bilirubin, hemoglobin • calcein fluorometry • EGTA

The complexometric titration of calcium, first introduced by Biedermann and Schwarzenbach (1), is one of the simpler techniques routinely used in the clinical laboratory. However, one objectionable feature of this technique is the obscure endpoint obtained with various metallochromic indicators. In addition, abnormal concentrations of hemoglobin or bilirubin interfere with the endpoint obtained in the titration of serum. "Calcein," a derivative of fluorescein introduced by Diehl and Ellingboe (2), forms a yellow-green complex with calcium ions that permits the choice of a visual or fluorescent endpoint. The use of calcein and other metallochromic indicators for the complexometric titration of calcium has been reviewed by Diehl (3).

The difficulty encountered in obtaining a sharp visual endpoint prompted a number of investigators to use photometric (4–8) and fluorometric (9–12) instruments for defining the endpoint. The precision of analysis has been further improved through the use of a motorized buret for delivering the complexing agent, coupled with a meter or recorder for measurement of the endpoint (6–12).

I have evaluated a commercially available automatic titrator that incorporates a motorized buret coupled with a fluorometer and digital readout, which respond to the amount of complexing agent delivered. Calcein was used as the indicator and the calcium–calcein complex was quenched by using the chelating agent ethyleneglycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA).

Material and Methods

Instruments

The Fiske/Marius calcium titrator (Fiske Associates, Inc., Uxbridge, Mass. 01569) consists of a fluorometer, motorized buret, and amplifier coupled with necessary circuitry and switching functions for controlling the rate of titration (Figure 1). The volume of chelating agent necessary to quench the fluorescence is measured by continuous electrical impulses, which are counted on a digital impulse counter. Complete quenching of the fluorescent calcium–calcein complex stops delivery of the chelating agent from the buret. The fluorometer incorporates a tungsten exciter lamp with primary blue and secondary green interference filters and a photocell detector. The sensitivity of the fluorometer to endpoint quenching is adjustable and should be set at a maximum before operation. A magnetic stirrer built into the cuvet housing provides continuous mixing during the titration.

The analytical results obtained by using the Fiske/Marius calcium titrator were correlated with
calcium analyses obtained with the SMA 12/60 (Technicon Corp., Tarrytown, N.Y. 10591) and with the Model 403 atomic absorption spectrophotometer (AAS; Perkin-Elmer Corp., Norwalk, Conn. 06852).

Reagents

All reagents are analytical grade unless indicated otherwise. Water was distilled and de-ionized before use.

Potassium hydroxide, 1 mol/liter. A solution containing 56.0 g per liter.

Calcium solution. Dissolve 60 mg of calcium (G. Frederick Smith Chemical Co., Columbus, Ohio 43223) in 200 ml of water. Store in a brown bottle, in the refrigerator.

EGTA solution. Dissolve 400 mg of EGTA (G. Frederick Smith Chemical Co.) in 5 ml of 1M KOH and dilute to 1 liter with water.

Calcium standard, stock solution, 1 g of Ca per liter. Calcium carbonate (NBS Standard Reference Material no. 915, National Bureau of Standards, Washington, D.C. 20234) was dried overnight at 120°C and 2.5022 g was transferred to a 1-liter volumetric flask containing about 700 ml of water; 4.6 ml of concentrated HCl was added to dissolve the salt and water added to the mark. The solution was stored in a glass bottle.

Calcium standard, working solution, 100 mg of Ca per liter. The stock standard was diluted 10-fold with water and stored in a glass bottle.

Procedures

Fiske/Marius calcium titrator. With the buret control switch in the “stand by” (pipet) position, press the “fill” switch and prime the buret with EGTA solution until air bubbles have been removed from the system. Place the buret switch in “ti-

trate” position and transfer 12 ml of 1M KOH and 0.1 ml of calcein solution to a clean cuvet. Place a stirring bar in the cuvet and transfer 100 µl of sample to the cuvet. In this evaluation I used two Lang/Levy self-adjusting pipets calibrated to contain 100 µl. Lowering the buret tip into the cuvet closes a switch, which starts the titration. A front panel indicator light goes on when the titration is complete. Elevate the buret tip and transfer the next specimen to the KOH solution. I was able to titrate 20 successive specimens with one filling of KOH. As the amounts of protein and chromogen increase, the titration time increases. The titration of 100 µl of serum or standard containing about 10 mg of calcium requires 1 to 2 min and gives a total count of about 500. The first titration usually gives an invalid endpoint, and should be ignored. Standard and sera were titrated in duplicate in this evaluation.

Calculation:

\[
\text{total counts for unknown} \times \frac{\text{concentration of standard}}{\text{total counts for standard}} = \text{concentration of unknown}
\]

SMA 12/60. Analyses for calcium were performed by the standard methodology, a modification of the procedure of Kessler and Wolfman (13). The SMA 12/60 was standardized with a commercial reference liquid having a reported value for calcium close to 10 mg/100 ml.

Results

The standard curve is shown in Figure 2. Values for sera from patients were calculated with the use of a single standard (10 mg/100 ml). A study of multiple titrations of this standard showed that the average of two titrations gave a reproducible value for use in calculating the value of the unknown. In the first month of this evaluation,
the standard was titrated 10 times each day; 214
titrations of the standard during this period gave
an average total count of 508 with a standard devi-
ation of ±4 counts (cv, 0.79%).

Precision

An evaluation of the precision of the calcium titr-
ator was based on duplicate analyses made by a
single analyst over a period of two months with
sera from inpatients. The standard deviation for
the analyses of 180 sera with a range of about 6
to 14 mg/100 ml was ±0.07 mg/100 ml (cv, 0.72%).
The same standard deviation was ob-
tained when 30 duplicate analyses were performed
within a single day. A larger coefficient of vari-
ation might be expected in laboratories where rou-
tine analyses are made on a daily basis and labora-
tory personnel are rotated.

Recovery

The recovery of calcium added to sera having
various initial concentrations is shown in Table 1.
Fifty microliters of standard (100 mg/100 ml) was
added to 5 ml of each serum to increase the total
concentration of calcium by 50 µg. The percent
recoveries in Table 1 and in other recovery studies
described below are calculated from an average of
four analyses on each serum.
The recovery of calcium from serial water dilu-
tions of a single serum is shown in Table 2.

Correlation Studies

In a study of the correlation between results
obtained with the calcium titrator and with the
SMA 12/60, 127 sera from patients were analyzed.
Sera were selected that had values ranging from
about 7 to 11 mg/100 ml. The scatter diagram
and regression line are shown in Figure 3. The
correlation coefficient for this comparison was
+0.918.
Results for the calcium titrator and AAS were
similarly compared for a second group of 128 sera
from patients. The scatter diagram for this
group is shown in Figure 4. The correlation coef-
ficient in this series of analyses was +0.985.

Interfering Factors

The effect of hemoglobin on the endpoint was
investigated by titration of a pooled serum con-
taining various amounts of hemoglobin. Hemo-
globin obtained from a whole blood hemolysate was
added to the pool to increase the hemoglobin con-
centration by 3 g/liter. Dilutions of this prepa-
ration were made with the original pool as diluent.
The results are shown in Table 3.
To determine the effect of bilirubin on the end-
point, I increased the total bilirubin concentration
of a single serum to 20 mg/100 ml by adding a
commercial preparation (“Dade Bilirubin Con-
trol,” Dade Division, American Hospital Supply
Corp., Miami, Fla. 33152) that contained no mea-
surable calcium. This serum was diluted with the
original serum and analyzed for calcium (Table 3).
The effect of increasing concentrations of mag-
nesium was determined by adding a solution of
magnesium sulfate to serum to increase the final
concentration by 1, 2, 4, 6, 8, and 10 mg/100 ml.
When these sera were titrated before and after
addition of magnesium, recoveries of calcium
ranged from 97.7% to 100.3% (av, 98.7%) with
individual standard deviations of less than ±0.9%.

Table 1. Recovery of Calcium Added to Six Sera

<table>
<thead>
<tr>
<th>Initial mg/100 ml</th>
<th>Expected mg/100 ml</th>
<th>Found mg/100 ml</th>
<th>Recovery, % (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.00</td>
<td>7.92</td>
<td>7.92</td>
<td>100.0 ± 4.2</td>
</tr>
<tr>
<td>7.55</td>
<td>8.47</td>
<td>8.46</td>
<td>98.9 ± 4.4</td>
</tr>
<tr>
<td>8.13</td>
<td>9.04</td>
<td>9.07</td>
<td>103.3 ± 4.3</td>
</tr>
<tr>
<td>9.19</td>
<td>10.09</td>
<td>10.12</td>
<td>103.3 ± 4.8</td>
</tr>
<tr>
<td>10.12</td>
<td>11.01</td>
<td>11.04</td>
<td>103.4 ± 4.3</td>
</tr>
<tr>
<td>10.87</td>
<td>11.75</td>
<td>11.76</td>
<td>101.1 ± 3.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean 101.7 ± 2.0</td>
</tr>
</tbody>
</table>

Table 2. Recovery of Calcium from Diluted Serum

<table>
<thead>
<tr>
<th>Calcium expected from dilution mg/100 ml</th>
<th>Calcium found by titration mg/100 ml</th>
<th>Recovery, % (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.50</td>
<td>9.46</td>
<td>99.6 ± 0.4</td>
</tr>
<tr>
<td>8.00</td>
<td>7.92</td>
<td>99.0 ± 0.7</td>
</tr>
<tr>
<td>6.00</td>
<td>6.07</td>
<td>101.2 ± 0.5</td>
</tr>
<tr>
<td>4.00</td>
<td>4.05</td>
<td>101.2 ± 1.1</td>
</tr>
<tr>
<td>2.00</td>
<td>1.81</td>
<td>90.5 ± 1.1</td>
</tr>
</tbody>
</table>

Initial calcium concentration: 10.00 mg/100 ml.

Table 3. Effect of Increasing Concentrations of Hemoglobin and Bilirubin on Titration of Calcium in Serum

<table>
<thead>
<tr>
<th>Added Hemoglobin mg/100 ml</th>
<th>Calcium found mg/100 ml</th>
<th>Recovery, % (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>...</td>
<td>8.76</td>
<td>...</td>
</tr>
<tr>
<td>30</td>
<td>8.76</td>
<td>100.0 ± 0.3</td>
</tr>
<tr>
<td>60</td>
<td>8.66</td>
<td>98.9 ± 0.4</td>
</tr>
<tr>
<td>100</td>
<td>8.68</td>
<td>99.1 ± 0.2</td>
</tr>
<tr>
<td>200</td>
<td>8.38</td>
<td>95.7 ± 0.7</td>
</tr>
<tr>
<td>300</td>
<td>8.00</td>
<td>91.3 ± 0.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Added Bilirubin mg/100 ml</th>
<th>Calcium found mg/100 ml</th>
<th>Recovery, % (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>...</td>
<td>9.98</td>
<td>...</td>
</tr>
<tr>
<td>1.0</td>
<td>9.98</td>
<td>100.0 ± 0.3</td>
</tr>
<tr>
<td>2.5</td>
<td>9.98</td>
<td>100.0 ± 0.5</td>
</tr>
<tr>
<td>5.0</td>
<td>9.82</td>
<td>98.4 ± 0.8</td>
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<tr>
<td>10.0</td>
<td>9.74</td>
<td>97.6 ± 1.1</td>
</tr>
<tr>
<td>15.0</td>
<td>9.47</td>
<td>94.9 ± 1.7</td>
</tr>
<tr>
<td>20.0</td>
<td>9.27</td>
<td>92.9 ± 4.5</td>
</tr>
</tbody>
</table>
Discussion

Since the complexometric titration of calcium was first introduced, investigators have searched for a metallochromic indicator that would permit the analyst to obtain a sensitive endpoint that would be easy to detect visually. It is doubtful that this goal has been achieved. Among the many indicators that have been used, calcein has been shown to give a fluorescent endpoint that is somewhat more sensitive than the endpoint obtained with nonfluorescent indicators (5). Some attempts have been made to improve the reproducibility of manual techniques by the use of some type of infusion pump or motorized buret, a fluorometer for detecting the endpoint, and a continuous recording device for monitoring the titration (9–12). The Fiske/Marius calcium titrator incorporates a motorized buret and fluorometer, but in addition features a controlled rate of titration and a digital readout that gives a numerical value proportional to the volume of complexing agent needed to reach an endpoint.

The precision obtained with this instrument depends almost entirely on the care used in measuring repetitive volumes of sample for analysis.

The results of recovery studies that use the calcium titrator are satisfactory for the range of calcium values most frequently obtained on sera from hospitalized inpatients (Tables 1 and 2).

The scatter diagrams and correlation coefficients (Figures 3 and 4) indicate that analyses of sera from patients by using the calcium titrator agree well with analyses of the same sera by use of the SMA 12/60 or AAS. The correlation coefficients (+0.918 for SMA 12/60, +0.985 for AAS) suggest that a slightly higher degree of correlation exists when the calcium titrator and AAS are compared.

There have been relatively few definitive studies reported on the interference of hemoglobin with the endpoint. By using manual techniques, several investigators (15, 16, 19) reported difficulty in seeing the endpoint when hemolyzed sera were being titrated, although they did not specify the actual hemoglobin concentrations. Using a semi-automatic technique and fluorescent endpoint, Cartier and Clement-Metral (9) reported no difference in the apparent total calcium concentrations when increasing amounts of whole blood hemolysate were added to serum. Actual concentrations of hemoglobin present were not stated. With the calcium titrator I obtained good recovery of calcium after hemoglobin was added in amounts as large as 100 mg/100 ml (Table 3).

Interference of bilirubin with the fluorescent endpoint is reportedly negligible at concentrations as large as 10 mg/100 ml (18), but larger concentrations give an increase in the amount of calcium apparently present. With the calcium titrator I obtained good recoveries of calcium after the addition of as much as 5 mg of bilirubin per 100 ml; with larger bilirubin concentrations, the apparent recovery of calcium decreased (Table 3).

Several investigators (15, 16, 19) have shown that magnesium does not interfere with the endpoint at concentrations well in excess of those obtained in hypermagnesemia. Borle and Briggs (12) used a semi-automatic titration assembly similar to the Fiske/Marius titrator and obtained essentially complete recovery of calcium from aqueous calcium solutions that were prepared with molar ratios of Mg/Ca up to 200. I found good recoveries of calcium when magnesium was added to serum in concentrations as great as 10 mg/100 ml.
If analytical technique is careful, use of the Fiske/Marius calcium titrator for routine analysis for calcium in serum should decrease analysis time and improve the precision of the results.

References

1. Biedermann, W., and Schwarzenbach, G., Complexometric determination of the alkaline earths and a few other metals with Eriochrome Black T. Chimia 2, 56 (1948).
3. Diehl, H., Calcein, Calmagite, and o,o'-Dihydroxyazobenzene. Titrimetric, Colorimetric, and Fluorimetric Reagents for Calcium and Magnesium. G. Frederick Smith Chemical Co., Columbus, Ohio, 1964.