Spectrophotometric Micro Method for Measurement of Dialyzable Calcium by Use of Cresolphthalein Complexone and Continuous-Flow Analysis

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We adapted a method for dialyzable calcium from fluorometric detection by use of calcein to a more specific spectrophotometric determination with cresolphthalein complexone. The reagents are available commercially and perform satisfactorily with respect to noise level, drift, stability, and sensitivity. Construction of the continuous-flow manifold with commercially available components (injection blocks, coils, and dialyzer) and the use of a 9-mm pathlength flow cell in an AutoAnalyzer I colorimeter have permitted a sampling rate of 70/h, and decreased the volume of serum required to 130 μL. A comparison of 71 sera analyzed by the present and the calcein method gave means of 1.39 (SD 0.14) and 1.39 (SD 0.13) mmol/L, respectively. The regression equation was: present method = 1.016 calcein − 0.022 mmol/L (r = 0.97). The CV for the new method, as determined from 46 randomized duplicate analyses, was <1%. The reference interval, as evaluated from results for 93 different individuals, was 1.26–1.43 mmol/L. We conclude that our method is an improvement with respect to noise level, drift, specificity, detectability, and more general availability of instrumentation. Moreover, the smaller sample volume required makes possible the routine measurement of dialyzable calcium in pediatric samples.

Additional Keyphrases: pediatric chemistry • fluorometry

Measurement of dialyzable calcium in either routinely processed serum (1) or anaerobically collected serum (2) has been shown to be more useful for most clinical purposes than is measurement of total calcium (2). In both of these methods for dialyzable calcium, fluorometric detection with calcein is used. In procedures for total calcium, this technique appears to be subject to interferences from some drugs, bilirubin, lipemia, and fatty acids (3). Also, pediatric samples reportedly give lower results relative to those by atomic absorption spectroscopy (3). Procedures for total calcium with dialysis in an acid medium and spectrophotometric detection by use of cresolphthalein complexone (CPC) (4) have few clinically important drug interferences (5), and results have been shown to correlate highly with those by atomic absorption methods (6, 7), one of which (7) is a reference method.

Our goal was to adapt the fluorometric dialyzable calcium procedure to the analytically more reliable methodology of CPC. We believed that the use of continuous-flow (SMAC, Technicon) manifold components might also allow the sample volume to be further decreased. Commercial instruments for measuring ionized calcium require about 0.5 mL per test, which often is more than is readily available for analysis of pediatric samples. A sample volume requirement of about 0.1 mL would be more practical for such patients.

We wished to use the same reagents, insofar as possible, for both the total calcium analysis (on the SMAC) and dialyzable calcium by our technique. Our results indicate that this and other modifications have improved the method with regard to sample requirement, noise, baseline drift, speed, and specificity.

Materials and Methods

Instrumentation

The flow system for dialyzable calcium (Figure 1) is assembled from a combination of AutoAnalyzer and SMAC components (Technicon Instruments Corp., Tarrytown, NY 10591). The dialyzer is submerged in a 37°C water bath. The debubbler is fabricated from a D2 connector with the curved arm bent to form a sideways "T". It must be mounted upright as shown in Figure 1. The debubbler-to-flow-cell connection tube is cut to a length of 5.0 cm. The flow cell is custom-made to have a pathlength of approximately 9 mm.

Reagents

Note: Use Straw and Filter Assemblies (Technicon no. 178-B651-P01) for all working reagents except Sample Buffer.

Sampler reservoir wash (Technicon no. T21-0778-85). Add 11 mL (5 mL/L) of wetting agent NWA (no. T21-0813-17) to 2.2 L of the wash solution.

Sample buffer. Prepare as described previously for Tris buffer, modified (2). It is very important to adjust pH accurately to 7.40 ± 0.01 at 37°C and to prepare fresh buffer if any growth or other debris forms.

Recipient. This reagent is similar to that described previously (2), except that it now contains 0.2 mL/L Triton X-100 instead of Triton X-405.

Cresolphthalein complexone (CPC) (no. T01-0709-82). Use as obtained from supplier.

Diethylamine (DEA) (no. T01-0867-82). Use as obtained from supplier.

Stock and working Ca standards are prepared as described previously (2).

Specimen Collection and Analysis

The collection, handling, and storage of samples are as described previously (2).

We find no need to prime the present system with a serum sample. The decrease in baseline drift allows the analysis of up to 20 samples between adjacent 1.50 mmol/L standards. Results may be calculated as reported previously (2), or by any other sufficiently precise technique.

Quality-Control Material

A human serum-based pool was prepared from sera remaining after five days' storage at 4°C. Grossly turbid or icteric samples were excluded. The serum was acidified by adding about 10 μL of 1 mol/L HCl per milliliter of serum. After stirring for about 1 min, the pool was filtered through
disposable serum filter columns (no. 11-387-50; Fisher Scientific Co., Pittsburgh, PA 15219) then injected into 3-mL Vacutainer Tubes. This process should be done quickly to avoid significant changes in pH of the serum pool. The tubes were stored at −20 °C until the day of analysis.

**Results**

**Manifold Design**

*Reaction time and temperature.* Upon combining the recipient stream with the CPC-DEA stream and mixing in a coil, we found that the resulting absorbances were virtually the same whether the mixing coil was maintained at room temperature (about 23 °C) or at 37 °C. Furthermore, there was little detectable difference in baseline noise when we used a shorter (10-turn) or longer (up to 30-turn) mixing coil. To minimize dwell time to about 2 min, we used a 10-turn coil at room temperature, for convenience.

*Sample/wash ratio.* We tried sample/wash ratios (in seconds) of 30/30, 40/20, and 35/16. Carryovers from aqueous-to-aqueous, aqueous-to-serum, and serum-to-serum samples were less than 5% with either the 40/20 or 35/16 ratios; therefore, we chose the 35/16 time (70/h cam: 2/1 sample/wash ratio) to decrease sample requirement and increase the rate of sample analysis to 70/h.

*Flow-cell length.* Use of the standard 15-mm AutoAnalyzer II flow cell gave an undesirably high absorbance reading (0.52 A or 30% T) for a 1.5 mmol/L Ca standard. Custom-made flow cells of 6 and 9 mm length were used, and the resulting absorbances were 0.25 A (56.5% T) and 0.33 A (46% T), respectively. For reasons of better detectability, less relative absorbance error, and apparently lower carryover, we chose the 9 mm flow cell.

*Segmentation frequency.* We found that, owing to the small grooves in the SMAC dialyzer, use of an SMA-type injection block for air-segmentation of the sample and recipient lines produced very long liquid/air segments through the dialyzer, resulting in imprecision. Segmentation with SMAC injection blocks produced a higher segmentation frequency with liquid/air segments of a smaller size, resulting in less carryover. We did not use the air bar on the pump module, because without it, the resulting bubble size gave satisfactory results and was similar to that produced by the SMAC instrument.

**Comparison of Results by Calcein and CPC Methods**

Results from the present spectrophotometric method were compared with those from the fluorometric method (2) for dialyzable calcium. During four days we analyzed 71 different serum samples. The results, summarized in Table 1, showed good agreement: the means were identical, and the average difference between results was 28 μmol/L. The regression equation indicates that there are small proportional and constant errors between the two methods.

**Precision**

Within-run precision was determined as the standard deviation of the difference between randomized duplicate
analyses of 46 different sera analyzed during three different days. The mean of the difference between any duplicate analysis was 10 (SD 9μL). The mean value for dialyzable calcium for the 46 samples was 1.26 mmol/L.

Between-run precision was determined from analyses on 11 days over a 29-day period. A human serum pool, prepared as in Materials and Methods, was used. The mean was 1.33 mmol/L (SD 9.1μL) and the CV was 0.7%.

Effect of PVP or PEG in Aqueous Standards

Continuous-flow acid dialysis methods for total calcium are complicated by differences in rates of dialysis of calcium ions between aqueous standards and serum. This is especially true if shorter dialyzers (12 in. or less) are used. Apparently, this is due to the resulting pH differential between aqueous standards and serum after each is mixed with an acidic diluent before dialysis. During dialysis, a difference in potential exists between sample and recipient streams, the magnitude of which depends on whether the sample is aqueous or serum (8, 9). Appropriate choice of acid concentrations in the diluent and recipient solutions and use of PVP in aqueous calcium standards reportedly minimize this discrepancy (9).

In our system we add Tris buffer to both standards and sera to maintain pH at around 7.40. Addition of either PVP (80 g/L) or PEG (50 g/L) to aqueous standards decreased the dialysis of calcium by about 6%. This is opposite to the effect of these polymers in aqueous solutions of calcium diluted with acid before dialysis (9). Evidently PVP and PEG have an affinity for calcium that retards its dialysis; this makes undesirable their use as additives to aqueous standards for dialyzable calcium. Furthermore, because the pH of sera or standards is nearly the same, the transmembrane potential causing differences in rates of ion exchange dialysis should be negligible.

Effect of Clotting Duration

Blood samples from four apparently healthy volunteers (ages 30 to 42 years) were used to study the effect that the duration of the interval allowed for clotting would have on the dialyzable calcium concentration. Samples were collected in five tubes from each person and the blood was allowed to clot in the sealed tubes for periods from 30 min to 6 h, then centrifuged and analyzed promptly. The results (Table 2) indicate that clotting time up to 6 h had no detectable effect upon results for dialyzable calcium.

Relative Permeability of Calcium Citrate

The original report on this method demonstrated that the rate at which calcium complexes cross the membrane is less than the rate for unassociated calcium ions (1). We determined the relative permeability of calcium citrate in the present system and found that this complex dialyzed only half as rapidly as did calcium ions (Table 3), although it dialyzed 60% as rapidly in the initial system (1). This percentage is based on the use of an association constant of 1280 L/mol for calcium citrate (10) to calculate the concentration of this chelate in the solution that was analyzed.

Results for Apparently Healthy Individuals

We collected serum as described from 46 men (ages 19 to 62 years) and 47 women (ages 21 to 65 years) who volunteered for this study. All were seated for 3–5 min before collection of blood. They had fasted for various intervals. The mean concentrations of dialyzable calcium were the same for each sex (1.34 mmol/L), but the reference range for women was wider (1.26–1.44 mmol/L) than for men (1.27–1.41 mmol/L). For all data, the mean and SD was 1.34 (SD 0.04) mmol/L. The data, when grouped according to age intervals of 10 years, had means that all were within the range 1.33 to 1.35 (SD 0.01) mmol/L.

Discussion

This adaptation of the method for dialyzable calcium from a fluorometric (1, 2) to a spectrophotometric technique has lessened the problems of baseline drift and of potential interferences in certain samples. Baseline drift was common in the method involving fluorometric detection with calcein (2), but is negligible when CPC is used for the analysis. In manual or non-dialysis calcein methods for total calcium, some drugs, bilirubin, and fatty acids reportedly interfere, and lower values are sometimes encountered in samples from pediatric patients and from those with renal disease (3). The proper application of CPC–DEA methods with acid dialysis to the analysis for total calcium has consistently produced excellent results in comparison with atomic absorption and reference methods (6, 7) and, therefore, is apparently more specific than is fluorometric detection with calcein.

Because the sample size requirement has been reduced from 280 μL to 130 μL, the method is more useful for pediatric samples, where the 0.5–mL samples of blood typically required for measurement of ionized calcium are often impracticable. Drawbacks to our method are the difficulties in collecting

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**Table 1. Fluorometric and Spectrophotometric Methods Compared**

<table>
<thead>
<tr>
<th>Method</th>
<th>Mean (SD), mmol/L</th>
</tr>
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<tbody>
<tr>
<td>Calcine fluorometric (x)</td>
<td>1.39 (0.13)</td>
</tr>
<tr>
<td>CPC spectrophotometric (y)</td>
<td>1.39 (0.14)</td>
</tr>
<tr>
<td>Difference (x − y)</td>
<td>0.026 (0.019)</td>
</tr>
</tbody>
</table>

Regression equation: $y = 1.018x - 0.022$ mmol/L.
Correction coefficient: 0.973.
Range: 1.06–1.80 mmol/L.
$n = 71$ for each method.

**Table 2. Effect of Clotting Duration on Dialyzable Calcium**

<table>
<thead>
<tr>
<th>Age, sex</th>
<th>Duration of clotting, h</th>
<th>Dialyzable Ca, mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>37, 9</td>
<td>0.5</td>
<td>1.33</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.33</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.33</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.32</td>
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<tr>
<td></td>
<td>4</td>
<td>1.33</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1.33</td>
</tr>
</tbody>
</table>

**Table 3. Relative Permeability of Calcium Citrate**

<table>
<thead>
<tr>
<th>Solution components, mmol/L</th>
<th>Calcium citrate</th>
<th>Ionic calcium</th>
<th>Dialyzable calcium</th>
<th>Relative permeability</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. 1.50 CaCl$_2$</td>
<td>0.15</td>
<td>1.50</td>
<td>1.50</td>
<td>1.00</td>
</tr>
<tr>
<td>150 NaCl</td>
<td>0</td>
<td>1.50</td>
<td>1.50</td>
<td>1.00</td>
</tr>
<tr>
<td>10 Tris (pH 7.4)</td>
<td>0.13</td>
<td>1.37</td>
<td>1.435</td>
<td>0.50</td>
</tr>
<tr>
<td>B. 1.50 CaCl$_2$</td>
<td>0.15</td>
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</tr>
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<td>0.13</td>
<td>1.37</td>
<td>1.435</td>
<td>0.50</td>
</tr>
<tr>
<td>0.20 citrate</td>
<td>1.50</td>
<td>1.50</td>
<td>1.50</td>
<td>1.00</td>
</tr>
<tr>
<td>10 Tris (pH 7.4)</td>
<td>0.13</td>
<td>1.37</td>
<td>1.435</td>
<td>0.50</td>
</tr>
</tbody>
</table>

*a Mean of four analyses.
*b Calculated with use of association constant.
*c Measured by present method.
small volumes of blood and the preparation of serum with minimal loss of CO₂. Heparinized whole blood cannot now be analyzed by this technique because the sample buffer contains a surfactant, necessary for proper flow of aqueous standards, that would lyse the erythrocytes. The addition of surfactant to standards and its elimination from the sample buffer might be a feasible approach to use of whole blood in the analysis. Construction of the manifold with SMAC injection blocks, mixing coils, and dialyzer permits use of smaller pump tubing, resulting in a smaller consumption of sample and reagents. This type of injection block minimizes carryover, apparently by increasing segmentation frequency. We now use a sampling rate of 70/h, with less carryover than in the other device, a 5% continuous-flow system (1, 2). A modified debubbler, shorter flow-cell, smaller tube diameter, and increased segmentation frequency apparently account for this better performance. The incorporation of a micro flowcell and electronic de-bubbling would further decrease carryover and might permit adaptation of this procedure to the SMAC. Assuming a sampling time of 23 s, sample volume required would diminish to about 85 μL. Apparently, the remaining drawback to use of this procedure on a multi-channel instrument is the requirement for anaerobic handling of specimens.

The present spectrophotometric method has greater detectability than the fluorometric procedure (1). Although many fluorometric techniques are very sensitive, calcein fluoresces strongly in the absence of calcium, requiring the attenuation of fluorometers used in this assay. The virtual elimination of drift along with this better detectability contributes to the better precision by the present method.

Our study of 93 different apparently healthy volunteers clearly indicates the reference interval (1.26–1.43 mmol/L) is lower than that found by fluorometric detection (1.30–1.47 mmol/L) (1, 2). Reasons for this difference may include: spectrophotometric detection with CPC, calibration in the adjustment of sample buffer to pH 7.40 (37°C), slightly lower permeability of calcium complexes in the present system, and geographic and social differences in the population studied. Our reference interval has about the same range between lower and upper bounds, 0.17 mmol/L, as has been reported for ionized calcium: 0.20 mmol/L (11), 0.14 mmol/L (12), and 0.16 mmol/L (13). This suggests that the diagnostic sensitivity of dialyzable calcium may be similar to that of ionized calcium. Studies are underway to establish the validity of this supposition.

Our results indicate that calcium complexes may be slightly less permeable in the present system. However, it is apparent that neither method detects all complexes. Dialyzable calcium by our method is a rate-dependent process, in that only about 5% of the free calcium ions, and about 2.5% of the calcium citrate pass into the recipient stream. Therefore, this measurement must be regarded as different from ultrafiltrable calcium, where relatively high pressures force about 100% of the calcium ions and complexes through a membrane. While classical theory suggests that this would favor clinical use of dialyzable calcium, being closer in concentration to ionized calcium, two recent reports on transfusion of citrated blood (14, 15) indicate that ionized calcium may not be the ultimate quantity for assessing calcium homeostasis.

References