The Worthington “Ultrafree” Device Evaluated for Determination of Ultrafiltrable Calcium in Serum

John Toffaletti,1 Dale Tompkins,1 and Gail Hoff2

We evaluated a commercially-available disposable device (“Ultrafree,” Worthington Diagnostics) for the anaerobic preparation of protein-free ultrafiltrates from serum for measurement of ultrafiltrable calcium. Sufficient filtrate for the analysis is obtained within 10 min from 0.2 to 0.4 mL of serum at room temperature. We assessed these ultrafilters with regard to permeability of calcium citrate, exclusion of proteins, frequency of leakage, and effect of temperature on results. Within-run and day-to-day coefficients of variation for human serum pools were 1.2 and 1.5%, respectively. Reference intervals (in mmol/L) for total (2.16–2.58), ultrafiltrable (1.44–1.67), dialyzable (1.25–1.41), and ionized (1.04–1.25) calcium have been determined for a healthy population of 69 women and 81 men, ages 18 to 65 years. The device appears to be the most practicable yet available for use in making this measurement.

Additional Keyphrases: reference intervals - low-cost methods for the laboratory - atomic absorption spectroscopy

Measurement of free ionized calcium in blood is generally believed to give the best clinical assessment of calcium metabolism, although many studies have reported few, if any, calcium-related cardiac effects during transfusions of citrated blood in which ionized calcium concentrations became extremely low (1–4). Measurement of ultrafiltrable calcium (protein-free ionized plus complexed calcium) might provide a better index of the true physiological status, but because of technical limitations of older ultrafiltration procedures as well as a perceived lesser physiological importance of ultrafiltrable calcium there are few reports related to the measurement of ultrafiltrable calcium.

A disposable anaerobic ultrafiltration device (“Ultrafree,” Worthington Diagnostics, Div. of Millipore Corp., Freehold, NJ 07728) has recently become available with which a protein-free ultrafiltrate can be produced within 10 min from 200–400 μL of anaerobically-processed serum. No additional equipment is required other than for measuring total calcium. The reliability of the technique therefore depends upon the integrity of ultrafilter and the reliability of the procedure for total calcium. From a practical standpoint, any laboratory can now measure ultrafiltrable calcium at a nominal cost, whereas the measurement of ionized calcium may cost more than many laboratories can justify based on clinical use.

We have evaluated this device with regard to temperature effects, molecular permeability, precision, and other potential variables in the ultrafiltration process, and have determined reference intervals for total, ultrafiltrable, dialyzable, and ionized calcium from data on 150 healthy individuals, ages 18 to 65 years.

Materials and Methods

Subjects. The subjects, all volunteers, were employees of the laboratory and hospital or blood donors. They were selected with an eye to a broad distribution by age and sex. Results for one subject who later was found to be hypertensive were discarded. A group of six results for ionized calcium were not included, because they obviously were low (0.87–0.94 mmol/L) for technical reasons during that particular day. Three abnormally high results for ultrafiltrable calcium obtained early in the study were discarded on the basis that leakage of protein through the filter probably accounted for them.

Analytical methods. Procedures for total calcium (5), dialyzable calcium (6), and ionized calcium (7) were as described previously. Ultrafiltrable calcium was determined with use of Ultrafree filters attached to 1-mL syringes. Positive pressure was applied to the syringe plunger by an auto-injector and ultrafiltrate was collected in the self-contained reservoir at the top of the Ultrafree device. Both ultrafilter and auto-injector are available from Worthington Diagnostics. Between 200 and 400 μL of serum was collected into the syringe for the ultrafiltration. After 100–150 μL of ultrafiltrate was collected (at room temperature), it was analyzed for calcium content by atomic absorption spectroscopy (5).

For measurement of ultrafiltrable, dialyzable, and ionized calcium, serum was collected with minimal exposure to air in 1-mL tuberculin syringes, as described previously (8). These syringes were capped and kept refrigerated or on ice until the analysis. All analyses were done within 8 h of collection.

Preparation of serum pool. A pooled specimen of human serum was prepared from patients’ samples left over from routine analysis and stored for three or four days at 4 °C. The pH was adjusted to about 7.4 by adding 10 μL of 1 mol/L HCl per milliliter of serum. After mixing, the serum was filtered, then injected into 3-mL evacuated blood-collection tubes. The tubes were stored at −20 °C and individually thawed on the day of analysis.

Results

Ultrafiltration of serum at room temperature and at 37 °C. Sera from 20 of the healthy volunteers were ultrafiltered at both room temperature (23 ± 1 °C) and at 37 °C. Capped syringes containing serum were kept in a room at 37 °C for 25 to 30 min before ultrafiltration was begun. Ultrafiltrates were transferred to plastic screw-top vials as soon as sufficient ultrafiltrate was obtained. The results (Table 1) show that ultrafiltrable calcium averaged 0.06 mmol/L less at 37 °C than at 23 °C. Individual differences due to temperature ranged from 0.01 to 0.14 mmol/L, with a CV of 49%.

Restriction of albumin and amylase. A human serum pool, prepared as described above, was used for this study. The albumin (Mr, 66 000) and amylase (Mr, 54 000) in this pool measured 39 g/L and 81 U/L. No detectable albumin or amylase passed through four different filters we tested; the limits of detection for these analyses were 1 g/L and 3 U/L.

Permeability of calcium citrate. Two solutions were prepared, each containing, per liter, 1.50 mmol of calcium, 150 mmol of NaCl, and 10 mmol of Tris, at pH 7.4. One of the so-

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lutions also contained 0.2 mmol of sodium citrate per liter, to chelate a portion of the calcium. Each solution was filtered through four different filters; the ultrafiltrable calcium measured the same, 1.50 mmol/L, in both solutions. Evidently all the calcium citrate passed through the membrane.

Constancy of ultrafiltrable calcium during ultrafiltration.

We wondered if a change in protein concentration in the sample during ultrafiltration caused a Donnan equilibrium change to affect the ultrafiltrable calcium. To study this we used four sera from different patients, collecting 600 µL of each serum in syringes, and beginning the ultrafiltration. When 300 µL of concentrated serum remained in the syringe, a new filter was attached and another ultrafiltrate was collected. Analysis of the first and second ultrafiltrates showed no definite changes in ultrafiltrable calcium concentration (Table 2).

Precision. A serum pool, prepared as described above, was collected into 20 syringes and each was filtered through an Ultrafree device. Analysis of these ultrafiltrates gave a mean value for calcium of 1.52 (SD 0.018) mmol/L and a CV of 1.2%.

Aliquots of another human serum pool were analyzed during a month, in which analyses were performed on 18 days. The mean ultrafiltrable calcium during this period was 1.58 (SD 0.024) mmol/L. The CV was 1.5%.

Reference intervals. Samples from the 150 different apparently healthy adults (Table 3) gave the reference intervals shown in Table 3, calculated from the mean ± 2SD and from values falling within the 2.5 and 97.5 percentiles.

Leakage through filters. The ultrafiltrates are ordinarily clear and colorless, but about one filter in 50 of those used in this evaluation gave a straw-colored ultrafiltrate and contained protein that apparently had leaked around the membrane. This problem can be eliminated in most of these situations by observing the first droplet of ultrafiltrate to pass through the membrane: if any color is seen, the syringe should be removed from the autoinjector, a new filter installed, and ultrafiltration restarted.

Discussion

The Worthington Ultrafree filter provides a simple and rapid means to separate ultrafiltrable calcium. Additional costs are small, because no new instrumentation is needed. In laboratories where the low volume would make it difficult to justify the cost of both instrumentation and reagents to maintain typical semi-automated Ca-ion-specific electrodes, the Ultrafree device may provide an economical alternative. Furthermore, virtually no additional space is required for the ultrafilters and auto-injectors.

The design of the device is especially well suited for determination of free calcium. Wasted serum or “dead space” is only about 50 µL and the sample is maintained nearly anaerobic during the ultrafiltration process. As a result, ultrafiltrable calcium can be measured in as little as 0.2 mL of serum in about 10 min. The use of oil is not necessary, as it was in an adaptation of the Worthington anticonvulsant drug filter to the measurement of ultrafiltrable calcium (9). As noted, we occasionally encountered problems with leakage of protein around the membranes, but by simply observing the color of the initial ultrafiltrate, errors due to this should be avoidable.

In this study the ultrafiltrations were done at room temperature. This is very convenient both in terms of time and operation conditions. Theoretically, calcium fractionation should be done at 37 °C, because the equilibrium can shift with temperature change. The importance of this in clinical diagnosis has not been established, although there have been several reports in which ionized calcium was measured at room temperature, apparently without obvious losses in diagnostic efficacy (10–12).

The discrepancy between ultrafiltrable and dialyzable calcium is only partly explainable. The method for dialyzable calcium detects about 50% of calcium citrate, as compared to 100% by Ultrafree. Considering the permeability of other complexes, this amounts to a discrepancy of about 0.07 mmol/L. The difference between measurement of ultrafiltrable calcium at room temperature and dialyzable calcium at 37 °C accounts for an additional 0.06 mmol/L. A small part of the remaining discrepancy, about 0.1 mmol/L, may possibly be due to the detection methods: spectrophotometry for dialyzable calcium, atomic absorption spectroscopy for ultrafiltrable calcium.

Possibly there are calcium complexes with relative molecular masses between 2000 and 20,000, that can permeate through the ultrafilter but are mostly excluded by the membrane used for dialyzable calcium. Peptides, phospholipids, or amino acids such as γ-carboxyglutamate may also be included in this group.

The speed, precision, and simplicity of the Ultrafree filtration device may encourage the clinical study of ultrafiltrable calcium in situations where ionized calcium has been

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**Table 1. Ultrafiltrable Calcium at Room Temperature (RT) and at 37 °C**

<table>
<thead>
<tr>
<th></th>
<th>CAUF (RT)</th>
<th>CAUF (37 °C)</th>
<th>Δ</th>
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</thead>
<tbody>
<tr>
<td>Mean, mmol/L</td>
<td>1.55</td>
<td>1.49</td>
<td>0.063</td>
</tr>
<tr>
<td>SD, mmol/L</td>
<td>0.055</td>
<td>0.055</td>
<td>0.031</td>
</tr>
<tr>
<td>CV, %</td>
<td>3.5</td>
<td>3.7</td>
<td>49</td>
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</tbody>
</table>

n = 20 different samples. Room temperature was 23 ± 1 °C

**Table 2. Ultrafiltrable Calcium Content of Four Samples of Serum before and after Concentration**

<table>
<thead>
<tr>
<th>Original serum</th>
<th>CAUF, mmol/L</th>
<th>Concd. serum</th>
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<tbody>
<tr>
<td></td>
<td>1.28</td>
<td>1.28</td>
</tr>
<tr>
<td></td>
<td>1.43</td>
<td>1.40</td>
</tr>
<tr>
<td></td>
<td>1.73</td>
<td>1.70</td>
</tr>
<tr>
<td></td>
<td>2.03</td>
<td>2.03</td>
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</tbody>
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* Ultrafiltrate collected as the serum was concentrated from 0.6 to 0.3 mL.

* Ultrafiltrate collected as the serum was further concentrated from 0.3 to 0.15 mL.

**Table 3. Demographic and Analytical Data for Reference Population**

<table>
<thead>
<tr>
<th>Age and sex distribution</th>
<th>Mean ± 2 SD</th>
<th>95th percentile</th>
<th>Reference Interval</th>
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<tbody>
<tr>
<td>Age, yr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18–29</td>
<td>2.16–2.58</td>
<td>2.18–2.58</td>
<td>2.16–2.58</td>
</tr>
<tr>
<td>30–39</td>
<td>2.16–2.58</td>
<td>2.18–2.58</td>
<td>2.16–2.58</td>
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<tr>
<td>40–49</td>
<td>2.16–2.58</td>
<td>2.18–2.58</td>
<td>2.16–2.58</td>
</tr>
<tr>
<td>50–59</td>
<td>2.16–2.58</td>
<td>2.18–2.58</td>
<td>2.16–2.58</td>
</tr>
<tr>
<td>60–65</td>
<td>2.16–2.58</td>
<td>2.18–2.58</td>
<td>2.16–2.58</td>
</tr>
<tr>
<td>No. men</td>
<td>27</td>
<td>24</td>
<td>12</td>
</tr>
<tr>
<td>No. women</td>
<td>25</td>
<td>21</td>
<td>10</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Calcium</th>
<th>Reference intervals, mmol/L</th>
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<tbody>
<tr>
<td>Ultrafiltrable calcium</td>
<td>1.44–1.67</td>
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<tr>
<td>Dialyzable calcium</td>
<td>1.25–1.40</td>
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<tr>
<td>Ionized calcium</td>
<td>1.04–1.25</td>
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measured previously. Such measurements may provide insight into the lack of symptomatic hypocalcemia in the presence of the very low concentrations of ionized calcium often observed during transfusion with blood containing citrate and phosphate (1–4). The low cost to initiate this procedure should also be attractive for laboratories that regard high initial cost and cost per test of instrumentation for ionized calcium as excessive.

References

An Optically Clear Hypercholesterolemic Hypertriglyceridemic Quality-Control Material Prepared from Animal Lipid Sources

Gary J. Proksch and Dean P. Bonderman

A hyperlipidemic control serum can be simply prepared from animal lipid sources. Beta- and pre-beta-lipoproteins containing cholesterol and triglyceride are removed from porcine serum by treatment with dextran sulfate and calcium ions. A triglyceride-rich fraction containing only trace amounts of cholesterol is isolated from chicken egg-yolks. The two fractions are then combined in 40 mmol/L sodium bicarbonate to give the desired values for cholesterol and triglyceride. The preparation is stabilized against surface denaturation during long-term storage at 5°C, perhaps for as long as two years, by adding 0.25 g of Triton X-100 surfactant per liter, and against an accidental exposure to short-term freezing by adding 10 g of sucrose per liter. We used this solution as a diluent to reconstitute lyophilized bovine serum. The resulting product, having been prepared from only animal sources, is free of hepatitis-associated constituents, and is remarkably clear, homogeneous, and stable. Results obtained with it are precise.

The association of increased concentrations of certain serum lipid constituents and increased risk of heart disease (1, 2) dictates that the precision and accuracy of lipid assays be carefully monitored. Pooled specimens of lyophilized human or animal serum are commonly used to evaluate and assure analytical performance. Hyperlipidemic human sera are expensive to prepare and sometimes difficult to obtain.

Recently we examined 22 lots of commercial human-serum-based quality-control materials for several hepatitis-related materials: HBsAg, HBsAb, HBcAb, and HAVAb (3). All were positive for one or more of these constituents.

In view of the health hazard involved in handling quality-control materials prepared from pooled human sera, we show how to prepare hyperlipidemic serum from animal sources, such serum is naturally free of hepatitis-associated constituents.

The concentration of lipids in the serum of herbivores such as horses or cattle, from which large amounts of serum can be readily obtained, is too low to be useful in monitoring lipid assays effectively (4). Because of the denaturation and insolubility of beta- and pre-beta-lipoproteins during lyophilization, serum products with a high lipid content generally reconstitute slowly, and the reconstituted fluid is often quite turbid (5). The lack of clarity and the inhomogeneity related to the uneven dispersion of insoluble material may decrease the usefulness of these products (6). A simple way is needed to increase the triglyceride and cholesterol concentrations of animal sera without adversely affecting the reconstitution rate or clarity of the product.

Recently, we described a stable human lipoprotein diluent for use in reconstituting lyophilized human serum for the preparation of clear, hyperlipidemic quality-control materials...