Separation and Quantitation of Serum Constituents Associated with Calcium by Gel Filtration

John Toffaletti,1 H. J. Gitelman,2 and John Savory1,2,3

Gel filtration of serum by use of polyacrylamide beads (Biogel P-2) separates total calcium into four distinct peaks: an initial peak, corresponding to protein-bound calcium; a second peak containing the calcium complexes of citrate, phosphate, lactate, and sulfate; a third peak containing calcium bicarbonate; and a fourth peak or trough corresponding to the serum ionic calcium. An eluent containing (per liter) 140 mmol of sodium, 1.10 mmol of calcium, 0.50 mmol of magnesium, and 10 mmol of 2-[(tris(hydroxymethyl)methyl)amino]ethanesulfonic acid (pH 7.40 at 37 °C) provides physiological conditions that determine the equilibria between these calcium components. Association constants determined under these conditions permit calculation of the expected concentrations of the calcium complexes in each tube of eluent, and these concentrations closely correspond to the amount of bound calcium measured experimentally. The mean distribution of calcium in healthy individuals, as determined by this method, is (per liter): calcium protein, 1.00 mmol; calcium complexes, 0.31 mmol; and ionic calcium, 1.07 mmol.

Additional Keyphrases: ionic and "bound" Ca • normal values • apparent association constants for complexes of Ca

Our understanding of the relative amounts of the various bound forms of calcium in serum is derived from the application of ultrafiltration to serum. This has shown that about 45% of the total calcium is retained in the nondiffusing protein material while the protein-free ultrafiltrate consists primarily of ionic calcium and a small portion (~10% of the total calcium) bond to substances that are also diffusible across the ultrafiltration membrane. Because the various moieties that bind calcium differ in molecular size, it should be possible to separate these substances by gel filtration. Furthermore, if the concentration of ionic calcium is maintained constant as well as the other conditions affecting the binding of calcium—such as pH, temperature, ionic strength, and magnesium ion concentration—then the amount of bound calcium should not change even though the sample is diluted during gel filtration.

Our studies examine the validity of this hypothesis by using gel filtration to measure the binding of calcium to substances of small molecular weight in serum. Knowledge of the association constants for several calcium complexes under physiological conditions allows us to predict the expected amounts of these complexes and to compare this to the amount measured by the gel filtration technique. Comparison of the association constants determined here with those reported elsewhere, as well as the agreement between the amounts of complexed calcium predicted and the amounts measured, indicate that gel filtration is a valid technique for identifying and estimating calcium complexes in serum.

Materials and Methods

Apparatus

All gel-filtration work was done with Glenco 3400 series columns, 1.5 × 100 cm, with water jackets (Glenco Scientific, Inc., Houston, Tex. 77007). A circulating water bath maintained the jacketed columns at 37 °C. Fractions were collected with a Fractomat Automatic Fraction Collector (Buchler Instruments, Fort Lee, N. J. 07024). The automated analyses were done with AutoAnalyzer modules: Fluoronephelometer, Colorimeters, Proportioning Pumps, and Sampler 2 (Technicon Instruments Corp., Tarrytown, N. Y. 10591).

Reagents

Biogel P-2 (100–200 mesh), Bio-Rad Laboratories, Richmond, Calif. 94804. For each column, 60 g of Biogel P-2 was swollen in the column eluent. This slurry was poured into the column and packed according to usual procedures.

Column eluent. The eluent contained, per liter, 140 mmol of sodium (130 mmol of NaCl, 6.5 mmol of NaOH, and 3.0 mmol of NaN3), 1.10 mmol of CaCl2, 0.50 mmol

1968 CLINICAL CHEMISTRY, Vol. 22, No. 12, 1976
of MgCl₂ and 10 mmol of 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid [N-tris(hydroxy-
methyl)methyl-2-aminoethanesulfonic acid buffer, TES; Calbiochem, La Jolla, Calif. 92037] adjusted to pH
7.40 at 37 °C (7.59 at 25 °C) with either HCl or NaOH solutions.

Methods

Automated procedures for calcium (1), total protein (2), phosphate (3), bicarbonate (4), and sulfate (5) were
used as reported or with minor modifications where appropriate. Citrate (6) and lactate (7) were determined
manually by enzymatic techniques. Potassium was measured by flame photometry.

Procedures

Collection and Preparation of Serum

Blood was collected from fasting healthy donors and allowed to clot in a sealed glass tube for 30 min, then
centrifuged at 3500 × g for 3 min. The serum was withdrawn into a plastic syringe and capped. The pH of
serum was adjusted by adding 10 µl of a 1 mol/liter TES buffer (pH 7.3) per 10 ml of serum to achieve a
final TES concentration of 10 mmol/liter. The final pH was adjusted by bubbling moist CO₂/O₂ (5/95 by vol)
through the serum until the pH was 7.40 ± 0.01 at 37 °C. All sera were added to the column within 30 min of this
adjustment.

Gel Filtration

We applied 3.5 ml of serum to the top of the gel bed and began elution at a flow rate of about 0.75 ml/min,
the flow rate we obtained under a pressure head of 90 cm of water. About 45 ml were eluted before the col-
clection was started. Then 40 fractions of about 3 ml each, were collected. Immediately after collection, the
contents of individual tubes were analyzed for total calcium. When appropriate, the contents of the tubes
were also analyzed for bicarbonate, citrate, phosphate, total protein, lactate, sulfate, and potassium.

Calculations

To determine the increment of bound calcium in each tube, we measured total calcium in the tube and sub-
tracted the eluant baseline calcium concentration. All increments corresponding to a single peak within the
eluent profile were added together. We converted this peak area to the concentration of that particular species
of bound calcium in serum by calculating a factor that corrects for the difference between the volume of serum
applied to the column and the volume of eluent collected in each tube. This factor for multiplying the area
of each calcium peak to give the serum concentration of bound calcium is as follows:

\[ X = \frac{T - 1.10}{A + B + C ± D} \]

where \( X \) = factor for converting peak area to concentrations, \( A = \) area of calcium–protein peak (mmol/
liter), \( B = \) area of calcium citrate, calcium phosphate, etc. peak (mmol/liter), \( C = \) area of calcium bicarbonate
peak (mmol/liter), \( D = \) area of ionic calcium (+) peak or (−) trough (mmol/liter), 1.10 = eluant baseline cal-
cium concentration (mmol/liter), and \( T = \) serum total calcium concentration (mmol/liter).

Results

Pattern of Elution

Preliminary experiments demonstrated that Biogel P-2 could separate calcium bound to protein from that bound
to substances of smaller molecular weight. A typical elution profile for Biogel P-2 is shown in Figure
1. The profile begins at an eluant baseline calcium concentration of 1.10 mmol/liter. The first calcium peak
is protein-bound calcium (\( V_e/V_0 = 1.00 \)). The next major calcium peak consists of the calcium complexes
of phosphate, citrate, sulfate, and lactate, whose relative elution volumes are shown in Table 1. The third calcium
peak consists primarily of calcium bicarbonate. The elution of ionic calcium from the serum (\( V_e/V_0 = 2.07 \))
is seen as a trough later in the elution profile. When the concentration of ionic calcium in serum is greater than
1.10 mmol/liter, it would elute as a positive peak.
The eluant composition was designed to maintain the equilibria between calcium species in serum during gel filtration by controlling the concentrations of ionic calcium, magnesium, ionic strength, temperature, and pH. To evaluate the adequacy of pH control, we measured the pH in all tubes after elution. The pH was maintained at 7.40 ± 0.01 in all tubes except in those that contained bicarbonate. The maximum change observed in these tubes was an increase of 0.12 pH unit. The flow rate, determined by measuring the mass of each fraction collected, varied less than 1% during the entire collection period and was therefore considered constant.

**Determination of Apparent Association Constants**

To examine whether the observed concentrations of citrate, bicarbonate, phosphate, lactate, and sulfate accounted for the bound calcium measured in the individual tubes, we determined the association constants for the calcium complexes of these compounds. To do this, we prepared aqueous solutions containing (per liter) 140 mmol of sodium, 1.40 mmol of calcium, 10 mmol of TES (pH 7.40), 5 mmol of potassium, and various concentrations of either citrate, bicarbonate, phosphate, lactate, or sulfate, such that the concentration of the calcium complex was from 0.1 to 0.2 mmol/liter. Each of these solutions was chromatographed in the same manner as serum. Calcium, potassium, and the anion of interest were measured in the appropriate tubes. Elution patterns for the calcium bicarbonate and calcium citrate solutions are shown in Figure 2. The concentration of bound calcium was measured in each tube and used to calculate the apparent association constants listed in Table 1.

**Calculated vs. Measured Concentrations of Complexed Calcium**

Three sera from different individuals were chromatographed with the eluant concentrations of calcium, phosphate, citrate, bicarbonate, lactate, and sulfate measured in the tubes. The previously determined association constants were used to calculate the concentrations of calcium phosphate, calcium citrate, etc., expected in each tube. The sum of these components in each tube was then compared to the complexed calcium that we had measured experimentally in each tube. Table 2 lists the sum of these components and their comparison to the experimentally measured amounts of bound calcium. In almost all cases, these two values agree within ±10 µmol/liter.

**Analytical Recoveries of Serum Constituents Chromatographed on the Column**

To determine whether the amounts of compounds added to the column corresponded to the amounts measured in the tubes, we determined the analytical recoveries of calcium, protein, citrate, bicarbonate, and phosphate for two different serum samples. This was done by comparing the total amount of each constituent we found in the tubes to the total amount that we had applied to the column. The recoveries were: calcium 96.3%, protein 106%, citrate 92%, bicarbonate 94%, and phosphate 96%.

**Study of Healthy Individuals**

The distributions of calcium determined in 10 healthy individuals by this method are shown in Table 3a. Calcium bicarbonate is evidently the major individual calcium complex in serum with a mean concentration of 0.14 mmol/liter. The other two major calcium complexes are with citrate and phosphate, which are present in

![Figure 2](image-url)
in mean concentrations of 100 and 60 μmol/liter, respectively. Calcium lactate is a minor component, even though lactate undoubtedly accumulates after collection of blood. Calcium sulfate is calculated to be less than 10 μmol/liter, which is below the limits of detectability for this gel filtration technique. Concentrations of protein-bound calcium and ionic calcium are 1.00 and 1.07 mmol/liter, respectively.

Discussion

The results indicate that gel filtration is a feasible technique for separating and measuring bound calcium in serum. This method, similar in concept to the Hummel and Dreyer technique for studying protein binding of small molecules (8), appears to give accurate results by controlling the conditions known to influence calcium binding to proteins, which are pH (9), ionic strength (10), temperature (11), magnesium ion (12), and calcium ion concentration.

Observations supporting the validity of the technique include the following: (a) the association constants we report in Table 1 agree with other reports of association constants for calcium citrate and calcium phosphate (13, 14), calcium bicarbonate (15, 16), calcium lactate (16, 17) and calcium sulfate (13, 18); (b) the amounts of bound calcium collected from serum in each fraction tube agree with the amounts expected from calculations made with use of the association constants; and (c) the total amounts of protein-bound calcium, ionic calcium, and complexed calcium obtained by gel filtration agree with those observed by other methods (16, 19). These data provide evidence that the factors that influence calcium equilibria in serum—such as pH, ionic strength, temperature, magnesium, and calcium concentrations—are maintained at physiological levels during column chromatography.

This method appears to be the first that directly measures the complexed calcium in serum. The complexes are eluted in two major peaks, one containing calcium phosphate, calcium citrate, calcium lactate and calcium sulfate, and the other consisting largely of calcium bicarbonate. Calcium bicarbonate, calcium citrate, and calcium phosphate are the major complexes; calcium lactate and calcium sulfate are minor components. Within the error of the method, the calculated sums of these complexes appear to account for all the measured complexed calcium. Other calcium complexes are present in concentrations too low to detect.

Our mean serum-complexed calcium concentration of 0.31 mmol/liter agrees well with both the 0.32 mmol/liter found by Moore (16) and the 0.26 mmol/liter found by Pedersen (19). Both of these authors reported calcium bicarbonate to be the major calcium complex in serum, an observation confirmed by our work. Moore (16), after calculating the contributions of the citrate, bicarbonate, phosphate, and lactate complexes, could not account for about 80 μmol of complexed calcium per liter, and he ascribed this remainder to calcium sulfate. Our data indicate calcium sulfate is in much smaller concentrations, less than 10 μmol/liter. Walser (14), in the only previous study of the individual calcium complexes in sera from healthy individuals, found a mean complexed calcium concentration of 0.16 mmol/liter. This apparently low value may be ascribed to the techniques used for measuring the total and ionic calcium in the serum ultrafiltrates. As noted by Moore (16), the estimation of complexed calcium by subtraction of the ionic calcium concentration from the ultrafiltrable calcium concentration is subject to large error, depending upon the analytical methods used to measure these quantities. Our gel-filtration procedure eliminates this error, which originates from intermethod variation, by using a single method to analyze the fractions of eluate for calcium.

The data in Tables 2 and 3 show that in healthy individuals most of the complexed calcium can be accounted for by the concentrations of bicarbonate, citrate, phosphate, and lactate. Other complexes are present in concentrations too low to detect.

We anticipate that complexes at concentrations less than 30 μmol/liter will be undetected by this method. The precision (CV) of the automated fluorometric calcium procedure is 0.62%. Thus, an increment of about 10 μmol/liter can be reliably detected in each fraction.

### Table 3. Concentrations of Calcium Fractions in 10 Apparently Healthy Individuals (mmol/liter)

<table>
<thead>
<tr>
<th>Subject</th>
<th>JT</th>
<th>DL</th>
<th>PD</th>
<th>SK</th>
<th>HG</th>
<th>PP</th>
<th>GP</th>
<th>DL</th>
<th>FA</th>
<th>RW</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protein-bound Ca</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.22</td>
<td>0.90</td>
<td>1.08</td>
<td>1.03</td>
<td>0.96</td>
<td>1.05</td>
<td>0.96</td>
<td>0.77</td>
<td>0.86</td>
<td>1.14</td>
<td>1.00 ± 0.14</td>
</tr>
<tr>
<td><strong>Ionic Ca</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.02</td>
<td>1.08</td>
<td>1.05</td>
<td>1.03</td>
<td>1.15</td>
<td>1.06</td>
<td>1.13</td>
<td>1.03</td>
<td>1.08</td>
<td>1.06</td>
<td>1.07 ± 0.04</td>
</tr>
<tr>
<td><strong>Ca bicarbonate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.15</td>
<td>0.12</td>
<td>0.12</td>
<td>0.11</td>
<td>0.15</td>
<td>0.17</td>
<td>0.11</td>
<td>0.20</td>
<td>0.15</td>
<td>0.16</td>
<td>0.14 ± 0.03</td>
</tr>
<tr>
<td><strong>Ca citrate, Ca phosphate, etc.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.12</td>
<td>0.16</td>
<td>0.11</td>
<td>0.17</td>
<td>0.13</td>
<td>0.19</td>
<td>0.18</td>
<td>0.27</td>
<td>0.15</td>
<td>0.20</td>
<td>0.17 ± 0.05</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.50</td>
<td>2.27</td>
<td>2.36</td>
<td>2.33</td>
<td>2.42</td>
<td>2.46</td>
<td>2.37</td>
<td>2.30</td>
<td>2.28</td>
<td>2.55</td>
<td>2.38 ± 0.10</td>
</tr>
</tbody>
</table>

#### a. Measured

#### b. Calculated

<table>
<thead>
<tr>
<th>Fraction</th>
<th>JT</th>
<th>DL</th>
<th>PD</th>
<th>SK</th>
<th>HG</th>
<th>PP</th>
<th>GP</th>
<th>DL</th>
<th>FA</th>
<th>RW</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ca citrate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.09 ± 0.08</td>
</tr>
<tr>
<td><strong>Ca phosphate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.05 ± 0.04</td>
</tr>
<tr>
<td><strong>Ca lactate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td><strong>Ca sulfate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.01 ± 0.01</td>
</tr>
</tbody>
</table>
tube. This corresponds to a concentration of about 30 µmol/liter in the original serum sample, if the complex is distributed in three collection tubes.

There is relatively little information available concerning the role of complexed calcium in calcium homeostasis, although at least two reports indicate it may be more interesting than previously believed. Moore (20), in a study of forty-two patients with advanced cirrhosis, found the complexed calcium to be significantly increased over normals. Citrate and phosphate could not account for this increase and apparently neither could bicarbonate. Pedersen (21), in studying the fluctuations of calcium fractions resulting from postural changes, incidentally noted an unexpected decrease in the complexed calcium concentration if the subject was standing. The physiological implications of these observations remain to be elucidated.

Our technique would be appropriate to use in studying the complexed calcium in such cases. An elution profile should display an unusual type or amount of complexed calcium, and by virtue of the separation of such complexes, provide assistance in identifying any new complexes that may be present.

This work was supported in part by NIH grants No. P01 AM08458 and 5 T01 AM05054, and American Cancer Society Institutional Grant No. IN15-0.

References