Improved Method for Calculating Calcium Fractions in Plasma: Reference Values and Effect of Menopause


Multiple linear regression analysis showed that total calcium was significantly and positively related to albumin, globulin, anion gap, and bicarbonate in plasma from 556 normal postmenopausal women. The residual constant of 1.21 mmol/L approximated the normal mean for ionized calcium in plasma. The coefficients derived from the multiple regression analysis were used to calculate ionized calcium from total calcium and ligand concentrations in a series of 105 clinical cases in whom ionized calcium was measured with the calcium electrode. The regression equation was \( y = 1.00x -0.0039 \text{ mmol/L} \), where \( x \) is measured and \( y \) calculated \( \text{Ca}^{2+} \) \( (r = 0.78, \ P < 0.001) \). The protein-bound, ultrafiltrable, and complexed fractions of the calcium were also calculated for plasma from 69 normal young men, 66 normal young women, and 305 normal postmenopausal women. The increased plasma calcium in the postmenopausal group was accounted for by an increase in the complexed fraction, due to increases in plasma bicarbonate and anion gap.

**Additional Keyphrases:** anion gap · bicarbonate · osteoporosis · protein-bound and free (ionized) calcium · reference interval

Although McLean and Hastings (1) demonstrated over 50 years ago that the ionized fraction of calcium in plasma is the physiologically active form, this fraction is still not widely measured, despite the availability of ion-selective electrodes. The total calcium in plasma continues to be measured, even though it gives a misleading impression when the plasma protein concentrations are abnormal, e.g., in the presence of malignancy and liver disease (2).

Most of the methods used to correct the plasma total calcium measurement for protein concentration involve multiplying the deviation of plasma albumin from its normal concentration by some coefficient and adjusting the measured plasma calcium accordingly (3–5). This has at least two flaws. First, use of the "adjusted" plasma calcium assumes that a constant amount of calcium will be bound to each unit of albumin, whereas the amount bound is in fact proportional to the total calcium concentration, owing to the law of mass action (7). Second, other relevant ligands in plasma may be as important as albumin, but they tend to be ignored.

Here, we examine the relationships between plasma calcium and the constituents of plasma that bind calcium, and propose an empirical formula for calculating the concentration of ionized calcium. This formula was derived from measurements made on 556 normal postmenopausal women and validated by direct measurement of total and ionized calcium in plasma samples having a wide range of concentrations, in 105 clinical cases.

**Subjects and Methods**

We studied four groups of subjects.

Group A was a cohort of normal postmenopausal women recruited by media advertisement for a study of bone loss after the menopause (8). More than a thousand women applied to take part, but about half were excluded because of a history of bone or joint disease or because they were being treated with diuretics or other agents that might affect calcium metabolism. From the remaining 557 subjects, ages 35–75 y, blood was collected after an overnight fast for multichannel analysis in the smac II (Technicon Corp, Tarrytown, NY) continuous-flow analyzer. One case was subsequently excluded because of a very high globulin concentration, 63 g/L.

Group B comprised 105 hospital patients under investigation for disorders of calcium metabolism, particularly osteoporosis and hypercalcaemia or hypocalcaemia, from whom blood was collected for simultaneous measurement of ionized calcium and multiple biochemical analysis.

Groups C and D consisted of 69 male volunteers (ages 22 to 53 y) and 66 premenopausal female volunteers (ages 16 to 45 y), respectively, who provided blood samples after an overnight fast.

The ionized calcium in blood was measured with a Radiometer ICA1 Ionized Calcium Analyzer (Radiometer, Copenhagen, Denmark); results were adjusted to values at pH 7.4. Methods used in the smac II were: bromcresol-green dye binding for albumin, the biuret method for total protein, colorimetry with phenolphthalein for bicarbonate, colorimetry with molybdate for phosphate, and ion-specific electrometry for the plasma electrolytes. The anion gap was calculated as the difference between the sum of sodium and potassium (cations) and chloride and bicarbonate (anions).

**Results**

Table 1 gives the mean (and SD) values for the variables measured in the four groups. The relatively normal value for mean calcium concentration in plasma in the clinical series (Group B) is fortuitous; the range of values was wide (2.10–2.75 mmol/L). The low mean plasma albumin in this group resulted from the inclusion of a number of cases of malignant disease.

In the 556 subjects in Group A, total plasma calcium was regressed simultaneously on all measured plasma anions

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that might bind calcium: albumin, globulin, bicarbonate, anion gap, and phosphate. All the regression coefficients were highly significant except for that with phosphate. Re-analysis of the data without plasma phosphate gave the following multiple-regression equation \( r = 0.62, P < 0.001 \):

\[
\text{Plasma calcium} = t\frac{P}{0.015 \times \text{albumin (g/L)}} + 12.1 < 0.001 \\
+ 0.0059 \times \text{globulin (g/L)} \\
+ 0.0081 \times \text{bicarbonate (mmol/L)} \\
+ 0.010 \times \text{anion gap (mmol/mL)} \\
+ 1.21 \times \text{TCa}
\]

(1)

The residual constant was so close to the anticipated concentration of ionized calcium (1.21 mmol/L at a total plasma calcium of 2.42 mmol/L) as to suggest that these empirical coefficients could actually be used to calculate the concentration of ionized calcium in plasma. The equation was therefore re-written as:

\[
\text{Ionized Ca} = \text{total Ca} - 0.015 \times \text{albumin (g/L)} - 0.0059 \times \text{globulin (g/L)} - 0.010 \times \text{anion gap (mmol/mL)} - 0.0091 \times \text{bicarbonate (mmol/mL)}
\]

(2)

We then applied this empirical equation to the 105 patients for whom actual measurements of ionized calcium were available, and regressed the calculated values on the measured values, with the following results: calculated Ca\(^{2+}\) = 1.83 \times \text{measured Ca}\(^{2+}\) - 1.05 (± 0.059) mmol/L.

The calculated and measured values correlated well \( r = 0.90, P < 0.001 \), but the size of the slope and of the intercept meant that the calculation underestimates ionized calcium at low values and overestimates it at high values.

The above calculation was based on the assumption that each unit of ligand binds a fixed amount of calcium. This would be true if the ionized calcium were constant, or fell within a narrow range in a homogeneous population (as in the 556 controls). In the presence of variations in ionized calcium, however, each unit of ligand will bind a relatively constant proportion of the total calcium rather than a constant amount (7). The correct formula would therefore be based on the proportion of total calcium bound by each unit of ligand. We therefore calculated the proportion of the total calcium bound by each unit of ligand from the mean total calcium in the whole group. Thus if the albumin coefficient (equations 1 and 2) is 0.015 mmol of calcium per gram of albumin, and the mean total calcium in the group is 2.42 mmol/L, then the mean binding of calcium by albumin is 0.015/2.42, or 0.61% of the total calcium (TCa) per gram of albumin. The corresponding proportional coefficients for globulin, bicarbonate, and anion gap were then used to derive a new equation for the calculation of ionized calcium as follows:

\[
\text{Ca}\(^{2+}\) = \text{TCa (mmol/L)} - 0.00613 \times \text{TCa} \times \text{albumin (g/L)} \\
- 0.00244 \times \text{TCa} \times \text{globulin (g/L)} \\
- 0.0043 \times \text{TCa} \times \text{anion gap (mmol/mL)} \\
- 0.00375 \times \text{TCa} \times \text{bicarbonate (mmol/mL)}
\]

(3)

With this formula, ionized calcium values \( y \) were calculated for the patients in Group B and then regressed on the measured values \( x \), with the following result (see Figure 1):

\[
y = 1.00x - 0.0039 (± 0.052) \text{mmol/L}; r = 0.78, P < 0.001.
\]

The proportional coefficients permit calculation of all the major calcium fractions in plasma. The ultrafiltrable fraction of the plasma calcium is the total minus the amount bound to albumin and globulin. The complexed fraction is the ultrafiltrable calcium minus the ionized fraction. Table 2 shows the means and limits of these fractions in the normal men and women.

Finally, we compared the means of the calculated calcium fractions in the 66 premenopausal women with those in 306 of the postmenopausal women who were within 10 years of the menopause (Table 3). The increased total plasma calcium of 0.05 mmol/L in the postmenopausal group is entirely accounted for by the increase in the complexed fraction, which in turn is accounted for by increases in the anion gap and bicarbonate concentrations.

**Discussion**

We have shown that the ionic calcium can be calculated rather accurately from measurements of albumin, globulin, "anion gap," and bicarbonate, by using empirical coefficients. Most previous corrections for binding of calcium in plasma have taken account of plasma proteins, generally...
Table 2. Mean Values and 95% Intervals for Plasma Calcium and Calculated Plasma Calcium Fractions (mmol/L) in Young Normal Men and Women

<table>
<thead>
<tr>
<th></th>
<th>Men (n = 68)</th>
<th>Women (n = 68)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium Mean</td>
<td>2.41</td>
<td>2.40</td>
</tr>
<tr>
<td>95% limits</td>
<td>2.27–2.55</td>
<td>2.24–2.56</td>
</tr>
<tr>
<td>Protein-bound</td>
<td>0.85</td>
<td>0.83</td>
</tr>
<tr>
<td>Ca, mmol/L</td>
<td>1.55</td>
<td>1.56</td>
</tr>
<tr>
<td>(mmol/L)</td>
<td>1.48–1.62</td>
<td>1.49–1.63</td>
</tr>
<tr>
<td>Ionized</td>
<td>1.18</td>
<td>1.20</td>
</tr>
<tr>
<td>Ca, mmol/L</td>
<td>1.10–1.26</td>
<td>1.14–1.29</td>
</tr>
<tr>
<td>Complexed</td>
<td>0.38</td>
<td>0.36</td>
</tr>
<tr>
<td>Ca, mmol/L</td>
<td>0.32–0.44</td>
<td>0.29–0.43</td>
</tr>
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</table>

albumin, and nothing else. It is clear, however, that globulins, bicarbonate, and "unidentified anions," taken together, are at least as important as albumin. By subtracting the proportion bound by these anions from the total plasma calcium, a value very close to the measured value for ionized calcium can be obtained.

Determination of the binding coefficients of anions for calcium in plasma would ideally be done on subjects with identical ionized-calcium concentrations, because the only variation in total calcium would then be ascribable to binding. Our group of postmenopausal women was as homogeneous as it could be; therefore, it provided an appropriate data base for the derivation of empirical binding coefficients. The coefficients determined on this group were then applied to a group of subjects with a wide range of plasma calcium concentrations. By recognizing that the proportion of the plasma calcium bound to each anion would vary with the anion concentrations, we were able to derive a formula for ionized calcium that very closely approximates the measured value, although it is subject to greater error because it involves measurement of five variables. This explains why, even though the slope is unity, the coefficient of correlation between the calculated and measured values is only 0.78. A simpler formula, based on absolute rather than proportional coefficients, yields a rather higher correlation coefficient, but the slope is far from unity and it is manifestly less accurate.

Another approach to the binding of calcium in plasma is to use the known dissociation constants of the relevant calcium salts and solve the equation with an iterative computer procedure (9). This requires, strictly speaking, a measurement of each individual relevant anion concentration—notably citrate—and depends on the validity of dissociation constants determined in aqueous systems. It is not an appropriate procedure in clinical practice, where our empirical formula clearly is preferable. However, no calculation can be as convincing as direct measurement if the latter is available.

Our calculated means and ranges of plasma ionized calcium in young normals are very comparable to those determined by ourselves and other workers by direct measurement (7, 10–16). Our mean calculated normal value for the ultrafiltrable fraction (1.56 mmol/L) compares well with earlier data and is the same as that reported by Toffaletti et al. (17) and Andersen et al. (18). Little attention has been paid in the past to the complexed fraction, although, as the difference between the ultrafiltrable and ionized fractions, it is subject to considerable error. Our figure of 0.36 mmol/L for young normal subjects is similar to that reported by most workers (9) but higher than some (18, 19) and lower than others (17).

The unexpected result that has emerged from our analysis is the strong presumption that the increase in plasma calcium that is seen at the menopause is ascribable to an increase in the complexed fraction, which in turn results from the increases in anion gap and bicarbonate—both of which have been noted before (20) but which have attracted little attention. The causes of these metabolic changes are unknown. We have found (and will be reporting elsewhere) that they are associated with a small increase in plasma sodium and a decline in plasma chloride, which have also been reported previously (20, 21).

Whatever the explanation of these biochemical changes at the menopause, they produce a small but highly significant increase in the calculated complexed fraction of the calcium in plasma, which is sufficient to explain the increase in plasma calcium at the menopause we originally reported (22, 23) and generally assumed to be due to an increase in the ionized fraction. We have now presented evidence that the menopausal rise in plasma calcium may be due to a rearrangement of the calcium distribution in the plasma, which would explain why there is no significant change in plasma 1,25-dihydroxy vitamin D₃ (24) [nor probably in parathyroid (25)] at the menopause or in response to estrogen therapy (26). Two other studies have shown a higher value for total calcium in old than in young women but no difference in the ionized fraction (10, 11). (However, the increase in plasma calcium with age was seen with the Technicon AutoAnalyzer procedure but not with atomic absorption spectrophotometry (10). On the other hand, an increase in the ionized calcium fraction at the menopause has been reported in one study (14) and a decrease in ionized calcium during treatment with estrogen in another (26). It is these changes, unaccompanied by changes in serum parathyrin, which have led to the hypothesis of a change in the calcium set-point of the parathyroid glands at the menopause (27). However, these observations take no account of the well-documented changes in bicarbonate and other anions at the menopause, which are bound to increase the complexed fraction (28).

Table 3. Calcium Fractions and Concentrations (Mean ± SE) of Relevant Ligands in Plasma of Premenopausal Women and of Postmenopausal Women within 10 Years of Menopause

<table>
<thead>
<tr>
<th>Variable</th>
<th>Premenopausal (n = 66)</th>
<th>Postmenopausal (n = 305)</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin, g/L</td>
<td>44.9 ± 0.35</td>
<td>44.0 ± 0.13</td>
<td>2.8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Globulin, g/L</td>
<td>28.1 ± 0.30</td>
<td>29.0 ± 0.19</td>
<td>2.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Anion gap, mmol/L</td>
<td>9.5 ± 0.25</td>
<td>12.1 ± 0.11</td>
<td>9.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Bicarbonate, mmol/L</td>
<td>28.2 ± 0.25</td>
<td>29.1 ± 0.11</td>
<td>3.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Calcium, mmol/L</td>
<td>2.40 ± 0.01</td>
<td>2.43 ± 0.0042</td>
<td>3.1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Ionized Ca, mmol/L</td>
<td>1.21 ± 0.0047</td>
<td>1.20 ± 0.0023</td>
<td>1.9</td>
<td>NS</td>
</tr>
<tr>
<td>Ultrafiltrable Ca, mmol/L</td>
<td>1.56 ± 0.0045</td>
<td>1.60 ± 0.0025</td>
<td>5.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Protein-bound Ca, mmol/L</td>
<td>0.83 ± 0.0086</td>
<td>0.83 ± 0.0031</td>
<td>0.1</td>
<td>NS</td>
</tr>
<tr>
<td>Complexed Ca, mmol/L</td>
<td>0.36 ± 0.0041</td>
<td>0.40 ± 0.0015</td>
<td>11.2</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Because the complexed calcium is part of the ultrafiltrable fraction, an increase in it of 0.03 mmol/L at the menopause increases the filtered load by 0.03 mmol per liter of glomerular filtrate—more than enough to account for the increase in obligatory calcium excretion of ~0.005 mmol per liter of glomerular filtrate (8), even if tubular reabsorption does not discriminate against the complexed fraction, as it may well do (18). The increase in obligatory calcium excretion at the menopause is itself sufficient to explain postmenopausal bone loss without invoking an alteration in the sensitivity of bone to parathyryn (29, 30) or an alteration in the parathyryn set-point for calcium (27). An increased calcium loss of 0.005 mmol per liter of glomerular filtrate at a glomerular filtration rate of 120 mmol/min represents a daily calcium loss of ~0.9 mmol of calcium, which is enough to account for the 1% annual loss of bone that occurs in postmenopausal women (8). We do not exclude the possibility of an increase in ionized calcium at the menopause, but we suspect that the change in the complexed fraction will prove to be of major significance.

Note added in proof: Dr. W. G. Robertson of the King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia, has provided the following more rigorous version of our second equation for ionized calcium:

\[
[Ca] = \frac{TCa - 0.01257[Calalbumin] + 0.0049[Calglobuline]}{1 + 0.01257[Ca]} - 1 + 0.0049[Ca]
\]

\[
\frac{0.0835[Ca] \text{anion gap}}{1 + 0.0835[Ca]} - 0.0759[Ca] \text{bicarbonate}
\]

where [Ca] represents the ionized calcium concentration. This equation can be solved by an iterative computer program, yields results very similar to our equation, and in no way alters any of the conclusions in this paper.

References