Two Bone Alkaline Phosphatase Assays Compared with Osteocalcin as a Marker of Bone Formation in Healthy Elderly Women

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Serum bone alkaline phosphatase (ALP; EC 3.1.3.1) was measured with a wheat germ agglutinin (WGA) precipitation assay and with a new IRMA in a group of healthy elderly women. Both assays were correlated with serum total ALP activity and with osteocalcin. The two bone ALP assays have comparable within- and between-run imprecisions (WGA assay within-run CVs 2.6–5.4% and between-run, 4.0–5.1%; IRMA within-run CV 5.0% and between-run, 3.2%). Comparison of the WGA precipitation assay (x) with the IRMA (y) demonstrated a correlation coefficient of 0.87 [Deming regression equation: y = (0.58 ± 0.02)x – (4.62 ± 0.45); n = 101; Sxy = 1.26; P <0.001]. Correlation studies with osteocalcin and total ALP showed correlation coefficients (all P <0.001) of 0.34 and 0.65, respectively, for the WGA precipitation assay and of 0.36 and 0.68, respectively, for the IRMA. We conclude that the two bone ALP assays have similar imprecision and that neither can be given preference over the other as a marker of bone turnover.

Indexing Terms: immunoradiometric assay/wheat germ agglutinin precipitation assay/osteoporosis

Bone tissue is constantly undergoing renewal in a process called remodeling. Bone is resorbed by osteoclasts and the osteoblasts refill the cavity with bone matrix (the osteoid), which is subsequently mineralized. When less bone is formed than resorbed, bone loss results and, ultimately, osteoporosis. Bone loss is an asymptomatic process unless bone fracture occurs. To diagnose alterations in bone remodeling and to gain insight into the dynamic process of bone metabolism, sensitive assays are needed.

Bone alkaline phosphatase (ALP; EC 3.1.3.1) and osteocalcin are synthesized by the osteoblasts and both are used as biochemical markers of bone turnover (1–4). In patients with high rates of bone turnover, significant and high correlations have been found between osteocalcin and bone ALP (5, 6). In normal women there is still a significant correlation, but the r values are lower (7). In a group of healthy elderly women at risk of osteoporosis we compared a recently developed two-site IRMA for bone ALP with a bone ALP assay by wheat germ agglutinin (WGA) precipitation. We compared analytical variation, performance, and costs of the two assays, and also compared these markers of osteoblast activity with the total ALP activity and with osteocalcin.

Subjects and Methods

Subjects

Subjects were 101 elderly women (70 years and older) participating in a study investigating the effect of vitamin D supplementation on bone mineral content and bone turnover (8). Baseline serum samples used in this study were stored at −70°C.

The protocol was approved by the hospital ethical committee. All participants gave informed consent.

Assay Methods

ALP. ALP was measured according to the recommendation of the International Federation of Clinical Chemistry at 30°C with p-nitrophenyl phosphate as substrate (9). The method was adapted to the Hitachi 737 analyzer (Boehringer Mannheim, Mannheim, Germany).

WGA precipitation assay. The WGA precipitation assay was carried out according to Behr and Barnert (10) with slight modifications. Briefly, incubate six parts of sample with one part of Triton X-100 solution [200 g/L, according to Rosalki and Foo (11, 12)] for 30 min at 37°C. Add six parts of WGA solution [WGA (Sigma Chemical Co., St. Louis, MO; L-9640), 3 g/L in distilled water; stable for 3 days at room temperature) and incubate once more for 30 min at 37°C to precipitate the bone fraction of ALP. Measure the residual ALP activity, after centrifugation (10 min, 1600g), in the supernate to obtain nonprecipitated activity (NPA). Because of a differential sensitivity of the ALP isoenzymes to precipitation by different batches of WGA, correct the bone ALP (B) as suggested by Sørensen (13):

\[ B = \frac{(F_1T) - \text{NPA}}{F_1 - F_b} \]

where T indicates the total ALP in the sample, and F1 and Fb indicate the fraction of liver and (residual) bone ALP activity, respectively, in the supernate after WGA precipitation. Determine the correction factors for each new batch of WGA.

For determination of Fb, we inactivated the bone fraction of serum of healthy men by heating the serum for 10 min at 56°C. The residual liver ALP activity (Tres) was treated with WGA as described above. F1 is then equal to NPA/Tres. In the sera used for determination of F1, no intestinal ALP was detected by electrophoresis.
For determination of $F_b$, in cord blood of term neonates, only the bone fraction of ALP activity is assumed to be present (14). After WGA precipitation of cord-blood, $F_b$ was calculated as NPA/T.

**IRMA.** The IRMA Tandem-R Ostase kits were a gift from Hybritech Europe S.A. (Liege, Belgium). The assay is a solid-phase, two-site IRMA. Briefly, the assay was carried out as follows: Bone ALP (100-µL specimen) and a radiolabeled monoclonal antibody (100 µL) are pipetted into a plastic tube and mixed. Subsequently, a plastic bead coated with a monoclonal antibody is added. After overnight incubation at 2–8°C, the bead is washed three times to remove unbound labeled antibody. The radioactivity bound to the solid phase is measured in a gamma counter for 1 min and is directly proportional to the concentration of bone ALP. Calibrators for the assay are included in the Tandem-R Ostase kits.

**Osteocalcin.** Osteocalcin was analyzed by a RIA method using a kit from Incstar Corp. (Stillwater, MN).

**Statistical Analysis**

Method comparison of the WGA precipitation assay and the IRMA for bone ALP was carried out by Deming regression analyses (15). Other correlation studies were carried out by least-squares regression analyses. The statistical significance of the difference between coefficients of correlation was calculated by transforming to normal deviates. All reported $P$ values are two-sided.

**Results**

**Imprecision of Bone ALP Assays**

The within-run imprecision of the WGA precipitation assay was 5.4% ($n = 10$) and 2.6% ($n = 12$) for two pooled sera with bone ALP activities of 14 and 76 U/L, respectively. The between-run imprecision was 5.1% ($n = 12$) and 4.0% ($n = 11$), respectively, for this pooled sera.

The within-run imprecision of the IRMA for bone ALP was 5.0% as determined from 210 duplicate assays (range 0–30 µg/L, mean 11.8 µg/L). The between-run imprecision was 3.2% ($n = 5$) for a sample with a mean concentration of 19.5 µg/L.

**Lot-to-Lot Variability of the WGA Assay for Bone ALP**

To check the lot-to-lot variability, we analyzed 50 serum samples with two different WGA batches. The mean bone ALP activities were 31.5 U/L (SD 15.8) and 32.4 U/L (SD 16.3) for the two batches. The orthogonal (Deming) regression equation was $y = (1.03 ± 0.02)x - (0.14 ± 0.58)$ ($r = 0.99, S_{yx} = 1.81$).

**Stability of the WGA Solution**

In spite of claims that the WGA solutions can be stored for a month at 2–8°C (10), in our hands this was not possible. After a few days of storage at 2–8°C, a visible precipitate formed in the solution. As expected, the capacity to precipitate bone ALP by this solution declined.

**Linearity of the WGA Assay**

Instead of using higher WGA concentrations for sera with high bone ALP activities, as suggested by Behr and Barnert (10), dilution of the sample was preferred. Using sodium chloride (9 g/L), phosphate-buffered saline pH 7.4, 50 mmol/L Tris-buffer, pH 7.4, or 50 g/L bovine (or human) albumin as a dilution medium yielded falsely high bone ALP activity. However, dilution with distilled water was satisfactory. A 1:1 dilution of samples up to a bone ALP activity of 300 U/L yielded the same result with and without dilution, so we concluded that the WGA precipitation assay was linear up to a bone ALP activity of 300 U/L.

**Specificity of the WGA Assay**

The specificity of the WGA assay depends on the precipitation behavior of the isoenzymes with WGA. Using the correction factors for $F_b$ and $F_T$, we found a bone ALP fraction in cord blood of 10.1% ($n = 10, SD 2$). In heat-denatured samples (10 min at 56°C) the residual liver ALP could not be precipitated with a 3.0 g/L WGA solution. To study the precipitation behavior of placental ALP, we used various sera from pregnant women. After denaturation of liver and bone ALP (5 min at 65°C) in these sera, the residual placental ALP could not be precipitated with a 3.0 g/L WGA solution.

**Hepatobiliary ALP** is also partly precipitated by WGA (10–13, 16). In samples containing hepatobiliary ALP as determined by electrophoresis, preincubation with Triton X-100 resulted in a clear reduction of the coprecipitation of this pseudobone ALP. Preincubation with Triton X-100 of samples without hepatobiliary ALP did not influence the results for bone ALP.

**Comparison of the Two Assays**

A comparison of the WGA precipitation assay ($x$) and the IRMA ($y$) for samples of 101 women >70 years is shown in Fig. 1. This comparison yields an orthogonal (Deming) regression equation of $y = (0.58 ± 0.02)x - (4.62 ± 0.45)$ ($n = 101; r = 0.87; S_{yx} = 1.26; P < 0.001$).

![Fig. 1. Correlation of bone ALP assays measured by a WGA precipitation assay and by an IRMA.](CLINICAL CHEMISTRY, Vol. 41, No. 2, 1995 197)
The mean WGA bone ALP was 28.3 U/L (SD 8.3) and the mean IRMA bone ALP was 11.7 µg/L (SD = 5.1).

Comparison of the Two Assays with Other Markers of Bone Metabolism

Comparison of the two bone ALP assays with osteocalcin and total ALP are shown in Fig. 2. Correlation studies of the WGA assay and the IRMA with osteocalcin yielded correlation coefficients of 0.34 \( (P < 0.001) \) and 0.36 \( (P < 0.001) \), respectively. With total ALP the \( r \) values were 0.65 \( (P < 0.001) \) and 0.68 \( (P < 0.001) \), respectively.

From correlation studies of total ALP with osteocalcin \( (n = 101) \) the correlation coefficient was 0.13 \( (P = 0.204) \). Nevertheless, this correlation coefficient was not significantly less than that for the bone ALP assays \((0.05 < P < 0.10)\).

Discussion

The WGA precipitation assay and the IRMA for bone ALP show comparable analytical imprecisions. Determination of bone-specific ALP by WGA precipitation is a simple method, but the major drawback is the difference in the amount of bone ALP precipitated by different batches of WGA (lot-to-lot variability). This can be circumvented by using correction factors determined for each batch of WGA.

The relatively short stability of the WGA solutions we saw (3 days at room temperature) does not agree with the results of others \((10, 13)\). Lot-to-lot variability can possibly be ascribed to this instability, leading to declining precipitation capacity of the WGA solution.

The WGA precipitation assay is linear to a bone ALP activity of at least 300 U/L. In our hands dilution of sera with higher activity with distilled water was adequate. Other solutions tested for dilution of the sera resulted in nonlinear precipitation behavior. A similar phenomenon was reported earlier \((17)\).

Cociprecitation of hepatobiliary ALP can at least partly be prevented by pretreatment of the serum with Triton-X 100. From our experiments, however, it could not be excluded that samples with a high activity of hepatobiliary ALP yield a residual activity of pseudobone ALP.

Recently a cross-reactivity of \(~16\%\) for liver ALP in the IRMA for bone ALP was reported \((18, 19)\). Because the mean nonbone ALP (total ALP minus bone ALP) is only 34 U/L, the influence of liver ALP on the IRMA results is rather small.

From the comparison of the WGA precipitation assay and the commercially available IRMA for measuring bone ALP activity we concluded that there is a good agreement between the two assays in these women at risk of osteoporosis. Although we studied only sera of healthy elderly women, a correlation coefficient of 0.87 was found.

The comparison of both assays for bone ALP with total ALP activity and with osteocalcin yielded no preference for one over the other. Although there is a significant correlation between both bone ALP assays and osteocalcin, the \( r \) values are rather low. This is in agreement with the \( r \) values found by Steinberg and Rogers \((7)\) in sera of normal women. In patients with high rates of bone turnover, higher \( r \) values were found for comparison of bone ALP with osteocalcin \((5, 6)\).

Both bone ALP assays showed a higher \( r \) value (although borderline significance) with osteocalcin than did total ALP with osteocalcin. This is in agreement with the results of Brixen et al. \((6)\).

In conclusion, both assays for bone ALP are easy to perform and show comparable intra- and interassay variation. No preference can be given for one assay over the other as a marker of bone turnover on the basis of analytical data. The net workload for both bone ALP assays is about the same. The overnight incubation of the IRMA is not a serious problem. The chemicals needed for the WGA assay are rather cheap, but the assay cannot be used for sera stored for longer than a month at \(-20^\circ C\), because the activity of ALP is not stable at this temperature. No special requirements are needed for the WGA assay, making this assay
applicable in every laboratory. The IRMA is like other immunoassays with radioactivity—expensive. For the performance of the IRMA some particular facilities are needed such as a special laboratory for using radioisotopes and gamma counting equipment. However, because the IRMA measures mass instead of activity it can be used for samples stored for a long time at \(-20^\circ C\).

References