Solid-Phase Enzymoimmunoassay for Osteocalcin in Human Serum or Plasma, with Use of a Monoclonal Antibody

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In this solid-phase enzymoimmunoassay on microtiter plates for osteocalcin in serum or plasma, we use an osteocalcin-horseradish-peroxidase conjugate and a monoclonal antibody raised against bovine osteocalcin. We thoroughly standardized the assay for measurement of osteocalcin in both serum and plasma, demonstrating independence of sample volume, and determining the analytical recovery and within- and between-assay CVs. The detection limit was between 0.6 and 1.1 μg/L and the ED₅₀ was 16 μg/L for a 5-μL sample volume. The intra-assay CV over the range 3 to 74 μg/L was ≤15%. The interassay CV over the range 3.6 to 46 μg/L was ≤16%. Results by this assay and by an in-house radioimmunoassay in which the same monoclonal antibody was used correlated well (r² = 0.948). Osteocalcin concentrations in serum and plasma as measured with the present assay agreed well with published values.

Additional Keyphrases: calcium-binding protein · radioimmunoassay compared · postmenopausal women · reference values · plasma vs serum as sample · metabolic bone disease · osteoporosis · bone gla protein

Osteocalcin is the most abundant noncollagenous protein in mature human bone, where it constitutes 1% to 2% of the total protein (3-5). Synthesized by osteoblasts (4, 5), it is incorporated into the bone matrix (6). Human osteocalcin is a 49-residue protein (M, 5669) with three gamma-carboxyglutamic acid residues, at positions 17, 21, and 24 (1). Although its function is not known, these three residues confer on it a very strong ability to bind to hydroxyapatite (7-9). Vitamin K₃ is essential for its biosynthesis (4, 10), which is stimulated by 1,25-dihydroxyvitamin D₃ (11-13).

Some of the newly synthesized osteocalcin escapes into the blood, and its concentration in serum reflects the rate of bone formation rather than the rate of bone resorption (14-17). It is a more specific biochemical index of bone metabolism than other more-established indices such as urinary hydroxyproline (18) and total serum alkaline phosphatase activity (19).

Serum osteocalcin measurements are used in various clinical situations, including metabolic bone diseases, renal disorders, hyperthyroidism, and diseases related to excess glucocorticoids (14, 20-22). Radioimmunoassays (RIAs) for osteocalcin have been described and are available commercially (23). Most are of conventional design, involving radioiodinated osteocalcin, polyclonal antibodies, and second antibody (24-26) or charcoal separation (27) steps. Inherent problems with RIAs include the relatively short half-life of the label, relatively long incubation times, difficulties in automation, and requirements for specialized facilities and equipment. Many of these problems may be overcome with enzymoimmunoassays (EIA).

An EIA for osteocalcin in which a polyclonal antibody and β-galactosidase-labeled osteocalcin are used was described recently (28). Here we describe a rapid, solid-phase EIA on microtiter plates for osteocalcin in which a monoclonal antibody and peroxidase-labeled osteocalcin are used. The assay has been standardized for both serum and plasma, and results correlated with those obtained with a published RIA.

Materials and Methods

Subjects

The 31 female control subjects, 20 to 90 years old, were in good health and were taking no drugs known to affect bone metabolism. Plasma and serum were separated from blood specimens and stored at -20 °C until assayed for osteocalcin—usually within two weeks but always within a year. In particular, for correlation studies, analytical recovery experiments, and dilution experiments, all samples were stored in aliquots to avoid repeated freezing and thawing and were assayed within four weeks of collection.

Reagents

Buffers: The general pH 7.4 assay buffer contained, per liter, 122.5 mmol of sodium chloride, 25 mmol of EDTA, 1 g of Tween 20 surfactant, 1 g of bovine serum albumin, and 10 mmol of Na₃HPO₄/NaH₂PO₄. Borate-buffered (pH 8.4-8.5) saline contained 75 mmol of sodium chloride, 100 mmol of boric acid, and 40 mmol of sodium borate per liter. Coating buffer was NaHCO₃/Na₂CO₃ (50 mmol/L, pH 9.6). Phosphate-buffered (pH 7.4) saline with bovine serum albumin contained 10 mmol of Na₃HPO₄/Na₂HPO₄, 150 mmol of NaCl, and 1 g of bovine serum albumin (Cohn Fraction V powder; Sigma, Poole, U.K.) per liter. Buffered substrate (pH 5.0) was a mixture of 2.5 L of 0.1 mol/L citric acid solution and 2.65 L of 0.2 mol/L Na₃HPO₄ buffer to which 5.9 mmol of H₂O₂ and 16 mmol of o-phenylenediamine were added per liter just before assay. All buffers contained thimerosal (100 μg/L) preservative and were stored at 4 °C. The washing solution was a 20-fold dilution of the following mixture in distilled water: 1.5 mol of NaCl and 5 mL of Tween 20 per liter. All buffer components were of high quality (analytical grade; Reidel De Haen, Hanoveryer, F.R.G.). Horseradish peroxidase (EC 1.11.1.7, Type VI) and 4,4'-ditiopyridine were from Sigma, Poole, Dorset, U.K. N-Succinimidyl-3-aminopropanesuccinate-3-carboxylate was supplied by CalBiochem-Behring Diagnostics, La Jolla, CA. Succinimidyl-4-(N-maleimidomethyl)cyclonexane-1-carboxylate was supplied by Pierce Chemical Co., Rockford, IL. Cyanogum bromide-activated Sepharose 4B and N-succinimidyl-3-(2-pyridyldithio)propionate were supplied by Pharmacia, Uppsala, Sweden. Flat-bottomed 96-well microtiter plates were from both Nunc A/S, Kamstrup, Denmark, and Costar Ltd., Cambridge, MA.

Osteocalcin: Bovine osteocalcin was purified from bovine bone (27). The final yield of purified osteocalcin was 12.1

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mg per 100 g of dried bone powder. Homogeneity of the purified osteocalcin was confirmed by electrophoresis on polyacrylamide gel and high-performance liquid chromatography (HPLC). Osteocalcin eluted as a single band (M, 13 000), as described by others (27). For HPLC we used a 150 × 4.6 mm column of Millipore Nova Pak C18 with an elution gradient from 0% to 70% acetonitrile in 1 g/L aqueous trifluoroacetic acid and an on-line integrator (Pye Unicam, CDPI computing integrator). Results indicated that the osteocalcin was 100.00% pure. Osteocalcin was quantified by the absorbance at 276 nm minus the absorbance at 320 nm, with use of 12.8 as the value for absorbivity (27). Osteocalcin stock standards (batch 6) were stored freeze-dried from assay buffer in aliquots of 4, 40, or 200 μg. Working solutions (0.625 to 80 μg/L) were stored at −20 °C in 0.5-mL aliquots and were used within one week after thawing.

**Antiserum:** Monoclonal antibodies were raised by standard procedures (29) with use of polyethylene glycol for the fusion procedure (30), as previously described (31). Sp2/0-Ag14 myeloma cells were grown on RPMI 1640 medium (Flow Laboratories, Irvine, Scotland) containing 200 mL of added fetal calf serum per liter (Gibco Laboratories, Paisley, Scotland), supplemented with glutamine (2 mmol/L), penicillin (kilo-int. units/L), streptomycin (100 mg/L), and amphotericin B (2.5 mg/L). Eight mice were immunized by injecting them with 50 μg of osteocalcin in Freund's complete adjuvant and the titer was boosted four times by two-weekly intraperitoneal injection of 10 μg of osteocalcin in Freund's incomplete adjuvant. Only one mouse, given conjugated osteocalcin, responded. The growth media of the viable clones were screened with use of an osteocalcin–peroxidase conjugate on 96-well microtiter plates. We detected 43 positive clones. After pre-selection and cloning, the best clone was selected on the basis of antibody titer, affinity, and growth characteristics of the cell line. Antibody titers were determined as described later. In subsequent large-scale culture, the cell line (72C8C8) produced a titer of approximately 1 in 500. However, this increased to 1 in 1400 after one cycle of freezing and thawing of the cell line. This antibody was used in the assay without further purification. The subclass of the monoclonal antibody used in this study was IgG1, as determined by both immunodiffusion studies and enzyme-linked immunosorbent assay.

Ascs fluid was produced in six- to eight-week-old mice by injection with 0.5 mL of pristane (2,6,10,14-tetramethylpentadecane), followed 10 days later with 2 × 10⁶ hybridoma cells. Ascs fluid was stored at −20 °C in 100-μL aliquots, or it was diluted 10-fold in borate-buffered saline and stored at 4 °C for analysis. The titer of the fluid ranged from 1 in 3.5 × 10⁵ to 1 in 1.6 × 10⁶, and the volume from 7 mL of ascites fluid for one sample to 100 μL for one sample.

Donkey anti-mouse IgG was obtained from Dako Laboratories, Glostrup, Denmark, and used without further purification.

**Osteocalcin–peroxidase conjugate:** Thiol groups were introduced into horseradish peroxidase by using N-succinimidyl-3-(2-pyridyldithio)propionate (32) or N-succinimidyl-S-acetylthioacetate (33), and the modified peroxidase was lyophilized and stored at 4 °C until required. Maleimide groups were introduced into osteocalcin by a procedure similar to that of Tanaka et al. (28). The number of thiol groups introduced into the peroxidase was estimated by use of 4,4′-dithiopyridine (32). The use of N-succinimidyl-S-acetylthioacetate and N-succinimidyl-3-(2-pyridyldithio)propionate resulted in the addition of 1.1 and 3.5 thiol groups, respectively, per peroxidase. Modified osteocalcin and peroxidase were mixed and Sephadex G-25 was added to absorb water and concentrate the reagents. The mixture was incubated at room temperature for 90 min, then overnight at 4 °C. Unconjugated osteocalcin and peroxidase were removed by gel filtration on Sephadex G-100 and ion-exchange chromatography on DE 53 anion-exchange resin. All fractions were monitored for osteocalcin by RIA (31) and for peroxidase activity by measuring at 403 nm. Fractions with the highest osteocalcin and peroxidase activity were pooled and stored at −20 °C.

**Serum**

**Osteocalcin-free serum:** We removed osteocalcin from serum or plasma by mixing with anti-osteocalcin antibody bound to cyanogen-bromide-activated Sepharose 4B (Pharmacia) prepared as described in the product leaflet. The antibody used in the immobilization was from the same hybridoma as the antibody used in the EIA, but was from ascites fluid rather than from culture medium. The binding efficiency of the antibody to the activated gel was 60% of the total protein, as measured by absorbance at 280 nm, and >99% of the total antibody, as measured by EIA. All serum and plasma samples, after an overnight incubation with this immobilized anti-osteocalcin antibody, contained less than 0.5 ng of osteocalcin per milliliter as measured by EIA or RIA.

**Quality-control sera:** Human plasma samples with low concentrations of osteocalcin were pooled and 0, 10, or 20 ng of standard osteocalcin (batch 6) was added per milliliter to portions of the pooled plasma to give controls containing 1, 11, or 21 ng/mL. Plasma or serum samples containing high, medium, or low concentrations of endogenous osteocalcin were pooled separately and used as supplementary controls. All quality-control sera were stored at −20 °C before use.

**Procedures**

**Well-to-well variation:** This was checked on both Costar and Nunc microtiter plates and eight-well strips. All 96 wells were coated with 200 μL of an appropriate dilution of rabbit IgG–peroxidase conjugate in coating buffer. After 90 min at 37 °C, the plates were emptied and unbound conjugate was removed by washing the wells three times with 300-μL portions of washing solution. Peroxidase activity was measured as described later for the osteocalcin assay. We assessed well-to-well variation with a BBC microcomputer, using the Platevar program (34).

**Coating of microtiter plates:** Each well was filled with 200 μL of donkey anti-mouse IgG, at a final dilution of 1000-fold, in coating buffer. Plates were covered with plastic film, incubated at 37 °C for 90 min, emptied, and washed to remove unbound antibodies. Monoclonal antibody, 100 μL, diluted in the albumin-containing phosphate-buffered saline, was added, and, after incubation at 37 °C for 60 min, the plate was washed as before. The plates then were stable for at least one month at 4 °C. For titration of media in the production of monoclonal antibodies we used the following protocol. Antibody dilutions ranging from 100- to 12 800-fold were prepared. Diluted antibodies were added to plate wells previously coated with donkey anti-mouse IgG. After 60 min, bound antibodies were detected by adding osteo-
calcine-peroxidase conjugate dissolved in assay buffer. After 3 h the plates were emptied and unbound conjugate was removed by washing the plates with washing solution. Peroxidase activity of the bound conjugate was measured as described in the osteocalcin EIA. The procedure for the detection of the monoclonal antibodies therefore included a step in which the mouse antisera were selectively removed from the medium by the second-antibody-coated solid phase (donkey anti-mouse IgG on microtiter plate). This eliminated the possibility of interference from osteocalcin present in the culture medium.

Osteocalcin enzymoimmunoassay: Pipette 5 μL of each standard solution into the appropriate wells, and 5 μL of buffer into the sample and control wells. Add 5 μL of osteocalcin-free serum to the standard wells and 5 μL of control, or sample, to the sample wells. Add 200 μL of an appropriate dilution, diluted in assay buffer, of osteocalcin-peroxidase conjugate (3 ng/mL as peroxidase) and mix gently. Incubate at 37 °C for 4 h. Wash the plate thoroughly and tap to remove excess moisture. Add 150 μL of the buffered enzyme substrate solution to each well and incubate at room temperature (16–19 °C). Stop the reaction after 30 min by adding 50 μL of 4 mol/L H₂SO₄ in the same sequence as the substrate solution was added. Mix the contents of the wells gently and measure the absorbance at 492 nm with a microtiter plate reader (Model EL307; Bio-Tek Instruments Inc., Burlington, VT). No problems were encountered with the use of 5-μL volumes because we used a positive-displacement pipette with a capacity of 5 to 25 μL (Boehringer Corp. Ltd., Dublin, Ireland).

Results
Well-to-Well Variation

The CVs for the six plates tested (three from Nunc and three from Costar), over the full 96 wells, ranged from 2.7% to 5.6%. No difference was detected between Nunc and Costar plates as regards the CV or the absorbance of the wells. Nor was there any difference between the CVs obtained for the middle 60 wells and the complete 96 wells in any microtiter plate. The outer 36 wells consistently gave higher absorbance than the inner 60 wells, and therefore we used only the inner 60 wells. A comparison of the 96-well microtiter plate with plates composed of 12 eight-well strips showed no difference in reproducibility or intensity of absorbance obtained.

Other Analytical Variables

Standard curve and precision profile: The standard curve (Figure 1) represents the mean from seven assays done during seven months. The detection limit—defined as the standard concentration equivalent to the mean absorbance for the zero standard (B₀) minus two times the SD of the B₀ values—for this set of standard curves ranged from 3 to 5.5 pg per well (0.6 to 1.1 μg/L). The ED₅₀ was 80 pg per well (16 μg/L). Figure 1 also shows the precision profile, representing the within-assay CV for four samples over the range of the standard curve.

Analytical recovery: We assessed the ability of the assay to quantify accurately osteocalcin standard added to plasma samples containing a range of endogenous osteocalcin concentrations. We added osteocalcin to four separate serum or plasma samples from four different subjects to give four different concentrations (Table 1). The total osteocalcin present was measured, and the recovery of added osteocalcin estimated after subtracting the value for endogenous osteocalcin. The overall mean recovery (n = 16) was 110% (SD 14.9%). This recovery experiment was repeated a month later, and the values were 106% (SD 15.2%).

Independence of volume: We examined the ability of the assay to measure osteocalcin in plasma or serum samples, independently of the volume of sample, testing four plasma and two serum samples, all from different subjects. These samples contained different endogenous osteocalcin concentrations, and volumes ranging from 2.5 to 20 μL were used. Results for the six samples were independent of volume.

### Table 1. Analytical Recovery of Osteocalcin Standard Added to Four Different Plasma and Serum Samples, as Measured by EIA with Monoclonal Antibody

<table>
<thead>
<tr>
<th>Added (μL)</th>
<th>Sample 1 (serum)</th>
<th>Sample 2 (plasma)</th>
<th>Sample 3 (serum)</th>
<th>Sample 4 (plasma)</th>
<th>Mean recovered (and % of added)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.5</td>
<td>24.5</td>
<td>31.5</td>
<td>39.2</td>
<td>—</td>
</tr>
<tr>
<td>12.5</td>
<td>20.0</td>
<td>39.5</td>
<td>45.0</td>
<td>49.4</td>
<td>13.2 (106)</td>
</tr>
<tr>
<td>25</td>
<td>28.0</td>
<td>57.5</td>
<td>61.2</td>
<td>71.2</td>
<td>29.2 (117)</td>
</tr>
<tr>
<td>50</td>
<td>56.2</td>
<td>87.5</td>
<td>93.7</td>
<td>92.4</td>
<td>57.0 (114)</td>
</tr>
<tr>
<td>100</td>
<td>111.2</td>
<td>132.5</td>
<td>139.5</td>
<td>124.9</td>
<td>102.0 (102)</td>
</tr>
</tbody>
</table>

CLINICAL CHEMISTRY, Vol. 35, No. 10, 1989
over the range tested, and over the range of the standard curve. Figure 2 shows results for four of these samples. When volumes were <1 µL, osteocalcin was overestimated, both in osteocalcin-stripped serum and plasma and in samples with low endogenous osteocalcin concentrations. We ascribed this to the promotion of antibody–conjugate binding by some factor or factors in serum or plasma. The effect was not eliminated by adding up to 50 mmol of calcium chloride or 200 mmol of EDTA per liter.

**Interassay variation:** Interassay CV was measured by repeated assay, over 10 standard curves, of four plasma samples with osteocalcin concentrations ranging from 3.6 to 46 µg/L. The CV was greatest at the lowest osteocalcin concentration, but CVs of 10% to 16% were readily attainable (Table 2). CVs of 10% to 11.8% were attained for three concentrations of osteocalcin in control samples, whereas the lowest concentration of osteocalcin (3.6 µg/L) gave a CV of 16% (Table 2).

The within-assay CV was ±15% over the range 15 to 370 pg per well (3–74 µg/L), which covers the range of the standard curve (Figure 1).

**Correlation with RIA**

Results correlated well for this EIA and an “in-house” RIA (31), performed with use of the same monoclonal antibody [r² = 0.948; the slope and intercept (±SE) were 1.21 ± 0.05 and 3.16 ± 0.89, respectively]. The EIA overestimated the osteocalcin concentration by 21% as compared with the RIA, as indicated by the slope on the correlation plot (Figure 3). There was also a slight tendency to overestimate in the analytical recovery experiments (110% and 106%). The reason for this overestimation is not known.

**Clinical Samples**

Osteocalcin concentrations in plasma samples from 31 female control subjects ranging in age from 20 to 90 years were measured with this EIA. The mean (±SE) osteocalcin concentration was 9.53 (±1.27) µg/L, with a median of 7.6 µg/L and a range from 1.0 to 25.5 µg/L (Table 3). These values are similar to those reported by others (35–38), but, as outlined previously (31), values for osteocalcin concentrations as measured by different assays depend on the antibody used in the assay.

**Discussion**

Several immunoassays for osteocalcin have been developed and standardized, but this enzymoimmunoassay has advantages over assays described hitherto. For example, the peroxidase-labeled osteocalcin is stable for at least two years at −20 °C and for eight weeks at 4 °C, which is a considerable advantage over 125I-labeled osteocalcin. Sample volumes of 2.5 to 20 µL may be used without prior dilution, in contrast to other published immunoassays in which larger volumes are needed (15, 20, 21, 27). Furthermore, in contrast to RIAs for osteocalcin, no special facilities for handling or disposal of radioactive isotopes are required.

In published immunoassays for osteocalcin, polyclonal antisera were used, whereas in this assay a monoclonal

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**Table 2. Inter-Assay Variation in Four Quality-Control Samples**

<table>
<thead>
<tr>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>3.6</td>
<td>0.6</td>
<td>16.0</td>
</tr>
<tr>
<td>8</td>
<td>10.5</td>
<td>1.1</td>
<td>10.0</td>
</tr>
<tr>
<td>10</td>
<td>22.6</td>
<td>2.6</td>
<td>11.8</td>
</tr>
<tr>
<td>10</td>
<td>46.8</td>
<td>4.7</td>
<td>10.3</td>
</tr>
</tbody>
</table>

**Fig. 3.** Correlation of osteocalcin concentrations measured in the same 36 plasma and serum samples by EIA and by a published, standardized, in-house RIA in which the same monoclonal antibody was used.

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<table>
<thead>
<tr>
<th>Age range, y</th>
<th>n</th>
<th>Median</th>
<th>Range</th>
<th>Mean</th>
<th>SD</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>20–40</td>
<td>13</td>
<td>12.5</td>
<td>1–22.5</td>
<td>12.6</td>
<td>7.7</td>
<td>2.1</td>
</tr>
<tr>
<td>&gt;60</td>
<td>18</td>
<td>5.2</td>
<td>1–25.5</td>
<td>7.3</td>
<td>5.8</td>
<td>1.4</td>
</tr>
<tr>
<td>All</td>
<td>31</td>
<td>7.6</td>
<td>1–25.5</td>
<td>9.5</td>
<td>7.1</td>
<td>1.2</td>
</tr>
</tbody>
</table>

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**Fig. 2.** Determination of the relationship between the volume assayed and the amount of osteocalcin detected for four serum (open symbols) or plasma (filled symbols) samples containing different osteocalcin concentrations.

The lines were fitted "by eye"
antibody is used. The epitope that this monoclonal antibody is detecting is important, because we and others have suggested that there are different forms of osteocalcin in human serum samples and that different antibodies do not recognize all of them equally.

The nature of the epitope recognized by this monoclonal antibody has not been determined. Most immunoassays for osteocalcin are calcium dependent, but this assay is calcium independent and should therefore facilitate epitope mapping, which is becoming more important as investigators attempt to standardize osteocalcin assays internationally. In preliminary unpublished results no cross-reactivity was detected between this monoclonal antibody and three peptides that contain amino acid sequences 38-49, 45-49, and 14-28 of osteocalcin. Likewise, Tanaka et al. (28), in an EIA for osteocalcin involving use of a polyclonal antiserum, did not detect cross-reactivity when osteocalcin was treated with trypsin, which cleaves osteocalcin at positions 19 and 43 of the intact molecule and so releases peptides 1-19, 20-43, and 44-49. If the antigenic site was located in these peptides, it would have been detected by this assay. Further studies are ongoing, but results to date suggest that the epitope may involve a specific conformation in the intact osteocalcin molecule.

This monoclonal antibody detected a protein that has the same molecular mass (6 kDa) as osteocalcin when serum containing high concentrations of osteocalcin was fractionated by passage through a Sephadex G-100 column (31). The same serum sample and the same protein fractions from the Sephadex G-100 column were analyzed by using two polyclonal antisera raised by us against bovine osteocalcin, and these also detected the aforementioned protein fraction corresponding to a molecular mass of 6 kDa (31). Values for serum osteocalcin in 36 samples determined with this monoclonal antibody were intermediate between those obtained in the same samples with the two polyclonal antisera 296B3A and 297BF (31), and relatively good correlations were obtained ($r^2 = 0.95$ and 0.91 for polyclonal antibodies 296B3A and 297BF, respectively). Also, the range of osteocalcin concentrations measured in 31 plasma samples with this monoclonal antibody (Table 3) were similar to published values. These results indicate that this monoclonal antibody compares favorably with polyclonal antibodies as regards its specificity and the range of osteocalcin concentrations it measures in serum.

The EIA overestimates osteocalcin by 10% when osteocalcin standard is added to serum and plasma samples. The only other published EIA for osteocalcin (29) underestimates the osteocalcin concentration present by about 10% (range 5-19%), when compared with an RIA in which the same antibody was used. This suggests that the measured osteocalcin concentration is influenced by the nature of the label or the batch of label. If this is so, it would be advantageous to use a stable label such as the peroxidase label used in this assay.

The range of osteocalcin concentrations measured in 31 serum and plasma samples was similar to published values (35-38). However, one must remember that the concentrations of osteocalcin measured with different assays are antibody-dependent (31).

Osteocalcin is the most promising bone-specific protein in serum yet investigated for the diagnosis and management of high-turnover metabolic bone diseases. The availability of standardized, cost-effective EIAs, done with use of monoclonal antibodies, should provide opportunities for confirming its applications as a predictor of rapid bone loss in diseases such as postmenopausal osteoporosis.

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