Avidin–Biotin Enzyme Immunoassay of Osteocalcin in Serum or Plasma

Jamal Jaouhari,1 François Schiele,1 Sylviane Dragacci,2 Pierrette Tarallo,1 Jean-Pascal Siest,2 Joseph Henny,1 and Gérard Siest1,3

We describe a competitive enzyme immunoassay, the ExtrAvidin®-biotin system, for determining osteocalcin in human serum or plasma. Antibodies were raised against bovine osteocalcin. Binding of the antibodies to osteocalcin was calcium-dependent. Limit of detection is 0.07 nmol/L (0.4 μg/L). The standard curve for method is linear between 0.3 and 17.6 nmol/L (1.9 and 100 μg/L). Interassay CV over the range 0.9 to 14.8 nmol/L (5.3 to 84 μg/L) is 7.5% to 11.7%. Analytical recovery is 105% ± 5% (mean ± SD). The measurement, which is adapted to microtiter plates, requires only 20 μL of serum and 5 h.

The coefficient of correlation between the concentrations measured by this method and by a commercially available radioimmunoassay kit (CIS Biointernational) is 0.91. Osteocalcin can be measured in serum or heparinized plasma. Hemolysis (174 μmol/L hemoglobin) reduces osteocalcin concentration by 54%. High concentrations of triglycerides (7 mmol/L) give an overestimation of 63%. Serum concentrations of osteocalcin measured in 130 healthy subjects (ages 15–64 years) and 86 children (ages 4–14 years) were 1.4 ± 0.8 and 4.0 ± 1.5 nmol/L (8.1 ± 4.6 and 22.5 ± 8.6 μg/L), respectively (mean ± SD).

Osteocalcin, also called bone Gla protein, is synthesized primarily by osteoblasts and in small amounts by odontoblasts (1). This 49-amino-acid polypeptide contains three γ-carboxyglutamic acid residues at positions 17, 21, and 24, with a molecular mass of 5669 Da (2). It is the most abundant noncollagenous protein in mature human bone, constituting 1% to 2% of the total protein content (1, 2). Ninety percent amino acid homology is found between bovine and human osteocalcin (2), and antibodies to bovine osteocalcin cross-react with human osteocalcin (2). It has a high affinity for hydroxyapatite and is chemotactic for a variety of cells. Its precise physiological function in the bone remains unclear, although several studies suggest that osteocalcin may regulate calcium or skeletal homeostasis (3).

Osteocalcin circulates in blood, and its concentration was measured in people with various metabolic bone diseases, including osteoporosis (4–7). These studies showed that circulating osteocalcin reflects the rate of bone turnover in most but not in all metabolic bone diseases in adults and that serum osteocalcin concentrations are consistently related to the rate of bone formation but not to that of bone resorption.

Radioimmunoassays (RIAs) of osteocalcin were first described by Price and Nishimoto (8), and some are available commercially (9). These methods use radioiodinated osteocalcin, polyclonal antibodies, or monoclonal antibodies (10) and need a separation step with a second antibody (8, 11) or with charcoal (3). RIAs have some disadvantages, including the relatively short half-life of the label, relatively long incubation times, difficulties in automation, requirement of specialized facilities and equipment, and the problems caused by the use of radioisotope.

Therefore, competitive enzyme immunoassays (EIAs) with polyclonal or monoclonal antibodies were developed, with osteocalcin coupled to β-D-galactosidase (EC 3.2.1.23) (12), alkaline phosphatase (EC 3.1.3.1) (13), or horseradish peroxidase (EC 1.11.1.7, HRP) (14). A competitive EIA with IgG conjugated to HRP was also published (15). The first EIA, published in 1986 by Tanaka et al. (12), was time consuming (29 h) and had a narrow range of linearity (0.9–0.4 nmol/L, 0.5–2.5 μg/L). The method with the IgG–HRP conjugate (18) was much more rapid but also had a small linearity range (0.1–0.9 nmol/L, 0.6–5 μg/L). In 1989, Power and Fotrell (14) published a method involving a monoclonal antibody. This method was characterized by a range of linearity from 0.4 to 14.1 nmol/L (2.2–80 μg/L) and could be performed in <5 h. It needed, however, the preparation and use of osteocalcin-free serum, and the use of ≥5 μL of sample (ratio of the sample volume to the reaction medium volume: 1:40) could be critical.

We describe here a simple and rapid competitive EIA involving biotinylated IgG antibodies to bovine osteocalcin. The ExtrAvidin®-biotin system leads to an acceptable sensitivity and a range of linearity from 0.3 to 17.6 nmol/L (1.9 to 100 μg/L) with 20 μL of sample.

Materials and Methods

Enzyme Immunoassay

Reagents. All reagents and biochemicals were of the highest grade available. Tween 20 and Tween 80 surfactants, ovalbumin, bovine serum albumin (BSA), ExtrAvidin-HRP, biotin-amidocaproate N-hydroxysuccinimide ester, o-phenylenediamine (OPD), bilirubin glucuronate, 2-[tris(hydroxymethyl)methyl]amino)ethanesulfonic acid (TES), and Sepharose–Protein G were from Sigma Chemical Co. (St. Louis, MO). Strip plates were from Costar Ltd.

1 Laboratoire du Centre de Médecine Préventive, 2 avenue du Doyen Jacques Parisot, 54000 Vandoeuvre-lès-Nancy, France.
2 Stabiligen SA, 30 rue Lionnois, 54000 Nancy, France.
3 Centre du Médicament, UA CNRS No. 597, 30 rue Lionnois, 54000 Nancy, France.
4 Nonstandard abbreviations: HRP, horseradish peroxidase; EIA, enzyme immunoassay; BSA, bovine serum albumin; HEPES, 2-(4-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; TES, 2-[tris(hydroxymethyl)methyl] amino)ethanesulfonic acid; and OPD, o-phenylenediamine.

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(Cambridge, MA). Tris, maleic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), citric acid, NaOH, free bilirubin, Na$_2$HPO$_4$, and Na$_2$HPO$_4$ were from Merck (Darmstadt, FRG). CaCl$_2$, H$_2$SO$_4$, NaCl, and glycero were from ProLabo (Paris, France). Intralipid$^\circledR$ was from Kabi Vitrum (Stockholm, Sweden) and 2-(N-Morpholino)ethanesulfonic acid (MES) from Boehringer Mannheim GmbH (Mannheim, FRG).

**Assay solutions.** The coating buffer (Tris saline buffer, pH 8.0) contained 10 mmol of Tris, 100 mmol of NaCl, and 20 mmol of CaCl$_2$ per liter. For the competitive binding step, we used Tris maleate buffer (pH 7.2) containing 100 mmol of Tris, 100 mmol of maleic acid, 5 mmol of CaCl$_2$, 10 g of ovalbumin, and 10 ml of Tween 80 per liter. ExtrAvidin-HRP was diluted in phosphate buffer (100 mmol/L, pH 7.2) containing ovalbumin (10 g/L) and Tween 80 (10 mL/L). The enzyme substrate was prepared by dissolving 31.5 mg of OPD in 10.5 mL of citrate buffer (citric acid 43 mmol/L, Na$_2$HPO$_4$ 140 mmol/L, and H$_2$O$_2$ 2 mL/L, pH 5.8). The washing solution was doubly distilled water containing Tween 20 (1 mL/L). All these solutions were prepared fresh each day.

**Osteocalcin.** Bovine osteocalcin dissolved in Tris buffer (Tris 10 mmol/L, NaCl 100 mmol/L, pH 8.0) was a kind gift from Stabiligen SA (Nancy, France). Osteocalcin was quantified at 280 nm with 1.33 g · L$^{-1}$ · cm$^{-1}$ as the absorptivity value (10). This solution was stable for 1 year when stored at $-20\degree$C in an equal volume of glycerol. The working solution (762 nmol/mL) was prepared each week by dilution in the Tris saline buffer.

**Antibodies.** Two Fauve de Bourgogne rabbits received a total of nine injections of 35.3 nmol (200 µg) of bovine osteocalcin at 20-day intervals. The antigen in 1 mL of isotonic saline solution with complete Freund’s adjuvant for the first injection and incomplete Freund’s adjuvant for the second one was injected subcutaneously at multiple sites for the two first injections and intramuscularly afterwards. By the fifth injection, the rabbits were bled from an ear vein 10 days after each injection. Antisera were lyophilized and stored at $-20\degree$C in 1-mL portions.

**IgG biotinylation.** The IgG fraction was purified by Protein G chromatography from the antisera with the highest titer. The protein in the purified IgG fraction was quantified with the method of Lowry et al. (16), with BSA as standard. The antibodies were biotinylated with biotin-avidinoprotein A-hydroxysuccinimide ester as described by Costello et al. (17). The biotin conjugate was stored at $-20\degree$C in glycerol (see above) and was stable for $>1$ year.

**Procedure.** We optimized the different steps of the immunoassay procedure. The concentration of the coated osteocalcin was tested to 26.5 nmol/L (150 µg/L). To choose the appropriate IgG-biotin and ExtrAvidin-HRP solutions, we used dilutions ranging from 1:1000 to 1:10 000 and from 1:1000 to 1:12 000, respectively. With an ExtrAvidin-HRP solution at a dilution of 1:400, the limiting range of the IgG-biotin fraction was 1:1000 (absorbance $>2$ units). Several buffers—Tris, Tris maleate, TES, and HEPES (each at pH 7.2), MES (pH 6.2), and succinic acid (pH 6.0), each at a concentration of 100 mmol/L—were compared in the competitive binding step. The duration of the incubation step was also studied.

The optimal procedure is as follows: Microtiter plates are coated with 100 µL of osteocalcin per well (8.8 nmol/L (50 µg/L) in the Tris saline buffer) and left to stand at 4 $\degree$C overnight in a humid chamber. The wells are then emptied, washed three times with the washing solution with a Tittertek Microplate Washer (S8112; Flow Laboratories, Helsinki, Finland), and tapped dry. The remaining reactive sites on the wells are saturated with 150 µL of the Tris maleate buffer containing ovalbumin and Tween 80, left to stand at room temperature for 30 min in a humid chamber, and then washed and tapped dry.

To each well are added 30 µL of the Tris maleate buffer, 20 µL of sample or standard (diluted serially in the Tris maleate buffer), and 50 µL of IgG-biotin (800 µg/L, corresponding to a dilution of 1:3000 in the Tris maleate buffer containing NaCl, 308 mmol/L). The plates are incubated on a microplate vibrator at room temperature for 2 h in a humid chamber and then washed three times and tapped dry.

ExtrAvidin-HRP (diluted 1:4500 in the phosphate buffer), 100 µL, is then added to each well. The plates are incubated at room temperature for 1 h in a humid chamber. After that, the plates are washed six times and tapped dry.

After addition to each well of 100 µL of the substrate (OPD) reagent, the plates are incubated at room temperature in the dark for 30 min. The reaction is stopped by adding 100 µL per well of H$_2$SO$_4$ (1 mol/L). The absorbance of each well is measured at 492 nm with an automated microplate reader (Tittertek Multiskan MCC/340; Flow Laboratories).

**Calculations.** Results were calculated by using a semilogarithmic graph. We used only the linear part of the standard curve to estimate osteocalcin concentrations. All unknown samples were assayed in duplicate.

**Characterization of the Method**

**Effect of calcium.** We compared the recoveries of osteocalcin measured with 0, 5, 10, and 20 mmol/L CaCl$_2$ in the competition medium. For each calcium concentration, we supplemented a fresh adult serum with a known amount of osteocalcin (range: 0.3–5.3 nmol/L, 1.9–30 µg/L). The effect of including 5 mmol/L EDTA in the standards and in some serum samples was also tested.

**Reproducibility and detection limit.** The interassay CV was calculated with pooled adult serum supplemented with three different concentrations of bovine osteocalcin: 0.9, 3.6, and 14.8 nmol/L (5.3, 20.6, and 84 µg/L). We assayed each supplemented sample in duplicate on nine different days.

The detection limit was defined as 3 SD of the blank value (absorbance measured in the absence of the osteocalcin). We calculated it as mean ± SD of six different daily measurements, made in quadruplicate each day.
Recovery and serum dilution. For studies of analytical recovery, we added known amounts of bovine osteocalcin from 0.2 to 5.3 nmol/L to three different serum samples from adults and children. The corresponding recovery was calculated as 100 × the ratio of measured osteocalcin concentrations to the quantity of added osteocalcin (taking into account the serum endogenous osteocalcin).

The dilution effect was studied with three different serum samples from adults and children supplemented with bovine osteocalcin (10.6 nmol/L) and diluted serially in the Tris maleate buffer.

Correlation with an RIA method. Serum osteocalcin concentrations in 68 healthy subjects (adults and children) were measured, both with this EIA method and with a commercially available RIA kit (CIS Biointernational, Gif sur Yvette, France).

Analytical interferences. The possible interferences of hemolysis, lipemia, and bilirubin were studied according to the recommendations of the Société Française de Biologie Clinique (18). We supplemented a pool of fresh serum from adults with free or conjugated bilirubin of 0 to 500 μmol/L. Hemoglobin was prepared from a fresh hemolsate as described by the Société (18). Hemoglobin concentration was measured with a Coulter Counter T600 (Coultronics France SA, Margency, France). The hemoglobin concentrations added to the serum ranged from 0 to 174 μmol/L. To evaluate the effects of lipemia, we added various concentrations of Intralipid (0–7 mmol/L of triglyceride) to the serum pool.

Effects of anticoagulants. We compared measured osteocalcin concentrations in 30 matched samples of serum and citrated or heparinized plasma from healthy subjects (adults and children). Several plasma samples with EDTA as anticoagulant were also assayed.

Human samples. We measured serum osteocalcin in 130 adults (ages 15–64 years) and 86 children (ages 4–14 years). All subjects attended the Center for Preventive Medicine in Vandoeuvre-les-Nancy (France) for a health examination.

Blood was collected into Vacutainer Tubes (Becton Dickinson, Grenoble, France). For serum, the 10-mL tubes contained a coagulation activator and a silicone gel. For plasma, we used 5-mL tubes with lyophilized lithium heparinate (143 USP units per tube) containing a silicone gel and 5-mL tubes with 500 μL of sodium citrate (129 mmol/L). The samples were centrifuged for 10 min at 1000 × g, and the concentration of osteocalcin was measured within the next 3 h. We also determined serum osteocalcin in two groups of hospitalized subjects: eight subjects (one man and seven women, ages 27–57 years) with Basedow disease, and 15 chronic alcoholic men (ages 28–51 years).

Statistics

All the results are given as mean ± 1 SD. The significance of differences between mean values was assessed by paired Student's t-test. Linear-regression analysis was used to evaluate the correlation between the EIA and RIA methods, and correlations between pairs of varietis were tested by using Pearson's coefficient.

Results

Effect of calcium. The best recovery was obtained with 5 mmol/L CaCl₂ in the Tris maleate buffer (Table 1). This addition was thus included in the procedure for measuring serum osteocalcin. The absorbance obtained for the 0.3 nmol/L osteocalcin standard was 1.36 A with 5 mmol/L calcium vs 1.28 A without calcium. With 5 mmol/L EDTA, the value of absorbance decreased to 0.48 A. Moreover, in several human serum samples the addition of 5 mmol/L EDTA resulted in very low absorbance values (<0.06 A).

Standard curve, detection limit, and reproducibility.

The standard curve (Figure 1) represents the mean of five measurements. The linearity for osteocalcin ranges from 0.3 to 17.6 mmol/L (1.9–100 μg/L), and the detection limit is 70 ± 50 pmol/L (0.4 ± 0.3 μg/L). The interassay coefficients of variation are 11.7%, 7.5%, and
Table 2. Analytical Recovery of Serum Osteocalcin

<table>
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<th>Measured osteocalcin, nmol/L</th>
<th>Recovery (x ± SD), %</th>
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<tr>
<td>Overall mean and SD</td>
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Fig. 2. Influence of dilution on serum osteocalcin measurement
The serum samples were diluted in 100 mmol/L Tris maleate buffer, containing 5 mmol of CaCl₂, 10 g of ovalbumin, and 10 mL of Tween 80 per liter, pH 7.2

8.9% for osteocalcin concentrations of 0.9, 3.6, and 14.8 nmol/L (5.3, 20.6, and 84 µg/L), respectively.

Analytical recovery and serum dilution. The mean recovery (Table 2) of different concentrations of osteocalcin (0.2–5.3 nmol/L) is 104.8% ± 5.0% (range: 96.9–111.9%). Serial dilutions of three different human sera (supplemented with bovine osteocalcin, 10–6 nmol/L) in the Tris maleate buffer gave acceptable results (Figure 2).

Correlation between the EIA and RIA methods. The EIA underestimates the measured osteocalcin concentration by 10% compared with the RIA (slope 0.90), and the intercept is near zero (Figure 3).

Analytical interferences. We observed a marked interference in serum samples supplemented with Intralipid (Figure 4A). A triglyceride concentration of 4.6 mmol/L increases the apparent measured serum osteocalcin by ~35%, which increases as much as 63% with 7 mmol/L triglyceride. Hemoglobin does not affect the measured osteocalcin in concentrations ≤35 µmol/L (Figure 4B). However, a significant decrease (~40%) occurs for a hemoglobin concentration of 87 µmol/L. The addition of 500 µmol/L free or conjugated bilirubin gives variations of ±15% in the measured serum osteocalcin concentration (Figure 4C). The influence of bilirubin does not appear to be related to the quantity of the added component.

Effect of anticoagulants. The osteocalcin concentrations determined in serum samples are 11.8% (not statistically significant) and 25.6% (P = 0.01) higher than those measured in heparinized and citrated plasma samples, respectively (Table 3). Osteocalcin was not measurable in plasma samples with EDTA as anticoagulant.

Physiological values. The average serum osteocalcin concentrations measured in samples from presumably healthy subjects are 1.4 ± 0.8 and 4.0 ± 1.5 nmol/L (8.1 ± 4.6 and 22.5 ± 8.6 µg/L) for adults and children, respectively. Data presented by age and sex in Table 4 show that serum osteocalcin concentrations are higher in children than in adults. In boys, the adult values are reached at 20 years, and in girls, at 15 years.

Hospitalized patients. The mean (SD) serum osteocalcin concentrations measured in patients with Basedow disease were 2.1(1.0) nmol/L and in chronic alcoholic men were 0.6(0.3) nmol/L.

Discussion

We developed a competitive EIA for the measurement of human serum osteocalcin by use of bovine osteocalcin and biotinylated IgG. Bovine osteocalcin is widely used for determining osteocalcin concentration in human serum because of its high cross-reactivity with the human protein (8) and its easier availability (9). Other RIAs based on osteocalcin of sheep (19) or human origin (20) have been published. However, until now, the
advantages of using antibodies to sheep or human osteocalcin were not, in our knowledge, clearly established. Osteocalcin exhibits conformational changes when calcium ions are bound to its Gla residues (21, 22). Therefore, the antigen–antibody reaction is sometimes calcium-dependent (12, 15, 23) and sometimes calcium-independent. In our assay, the addition of calcium ions is needed to yield a good recognition of the antigen; the best results for recovery are obtained with 5 mmol/L CaCl₂ (see Table 1). With higher concentrations of CaCl₂, the antigen–antibody binding might be modified by a possible ionic strength effect. Furthermore, the addition of 5 mmol/L EDTA to osteocalcin standards or to human serum samples leads to very low absorbance values, which suggest that osteocalcin is not recognized by the antibodies. Similarly, we were not able to measure plasma osteocalcin with EDTA as anticoagulant. These results strongly suggest that our assay system is calcium-dependent. This dependence is also corroborated by the fact that the use of citrate as anticoagulant, which is known to partly complex calcium, leads to a decrease in the measured osteocalcin (see below).

The use for the first time of the ExtrAvidin-biotin system for the determination of serum osteocalcin yields an acceptable sensitivity associated with a large linearity range from 0.3 to 17.6 nmol/L, corresponding to absorbance values of 1.36 to 0.3 A, respectively. In addition, blank values are very low (<0.07 A).

Among the published methods, the upper limit of linearity does not exceed 2.6 nmol/L (15 μg/L) except those of Pastoureau and Delmas (19) and Power et al. (23) for RIA and Power and Fottrell (14) for EIA, who obtained upper limits of linearity of 8.8 (19) and 14.1 nmol/L (14, 23) (Table 5). The detection limit of our assay is comparable with those given by others, although comparisons are difficult because of the use of different criteria for this calculation. The reproducibility is acceptable if one takes into account that the assay is performed manually and could certainly be improved if automated. The analytical recovery (104.8% ± 5.0%) of our method is satisfactory compared with recoveries of other methods (Table 5). No matrix effect was observed as assessed by the different dilutions of serum samples in Tris maleate buffer.

We studied potential interferences by lipemia, hemoglobin, and bilirubin. Lipemia >3 mmol/L triglyceride causes an overestimation of the measured osteocalcin by ~20%; it reaches 63% for 7 mmol/L triglyceride. This effect could be attributed in part to an alteration of the antigen–antibody reaction or to the possible binding of osteocalcin to phospholipid vesicles (24). Hemoglobin
decreases dramatically the apparent measured osteocalcin (for example, by 45% for 90 μmol/L hemoglobin). A decrease of 50% to 90% of the osteocalcin concentration in hemolyzed samples was reported by others, both for EIA (15) and RIA (10) methods. These authors suggested that the inhibition of osteocalcin immunoreactivity could be related to an enzymatic alteration in the osteocalcin or by binding to the erythrocyte membranes. The influence of free or conjugated bilirubin on osteocalcin determination is not clear (see Figure 4C). Addition of free bilirubin results in variations of the measured osteocalcin of ±15% without apparent relationship with the quantity of added free bilirubin. The effect of 50 to 500 μmol/L conjugated bilirubin is quite different: There is a decrease in the apparent measured osteocalcin concentration of ~15%. Some differences in the influence of the addition of free or conjugated bilirubin were previously reported for other serum components (25).

Human osteocalcin may be determined in either serum or heparinized plasma with our EIA, although values measured in serum are ~12% higher than those obtained in heparinized plasma (difference not statistically significant). These results agree with those obtained by others (10, 15, 23). Conversely, the use of citrate as anticoagulant appears not to be convenient, in our assay resulting in a 26% decrease (P < 0.05) of the measured osteocalcin, which is similar to the decrease of ~30% obtained by Power et al. (26).

The results obtained with our EIA correlate well with those obtained with a commercially available RIA kit. The EIA underestimates the osteocalcin by ~10% compared with the RIA kit from CIS Biointernational. Results given by various kits or published methods differ from each other (27), but the physiological values of serum osteocalcin measured with our method agree well with most of the published data (4, 6, 14). Maximum serum osteocalcin concentration is observed in children of ages 10–14 years. In girls, adult values are reached at 15 years, whereas in boys, they are reached about 5 years later. In adults 20–49 years old, there is no marked sex- or age-related change. However, after age 50, serum osteocalcin concentration increases more markedly in women than in men. These age and sex variations agree with those previously published (15, 28–30).

To validate our assay with what is known about osteocalcin concentration in some pathological states, we determined the protein in two groups of hospitalized subjects. We found a greater serum osteocalcin concentration in patients with Basedow disease than in healthy subjects, as expected (30). Conversely, serum osteocalcin concentration was shown to be decreased in chronic alcoholic subjects (31). Results obtained with our assay system—0.6 ± 0.3 nmol/L in chronic alcoholic men—confirm these findings.

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