Wheat-Germ Agglutinin Method for Measuring Bone and Liver Isoenzymes of Alkaline Phosphatase Assessed in Postmenopausal Osteoporosis
Steen Sørensen

In the method of Rosalki and Foo (Clin Chem 1984;30:1182-6) bone and liver isoenzymes of alkaline phosphatase (EC 3.1.3.1) are quantified by using wheat-germ agglutinin (WGA). I suggest standardizing the procedure by using a WGA concentration that precipitates half of the alkaline phosphatase activity of serum pooled from an equal number of healthy women and men. By applying knowledge of the precipitation pattern in serum samples containing predominantly or exclusively bone or liver sources of alkaline phosphatase, I obtained results for the isoenzymes in healthy subjects that agreed with those by the heat-inactivation methods, as reported earlier in the literature. I then assessed the utility of the standardized procedure in a clinical study of prevention of postmenopausal bone loss. In patients receiving hormone replacement therapy, which is known to decrease bone turnover, the decrease in total alkaline phosphatase activity in serum was entirely ascribable to decreases in the bone isoenzyme activity, probably reflecting reduced bone formation, whereas the activity concentration of liver alkaline phosphatase remained unchanged.

Additional Keyphrases: assay standardization · lectins · treatment with estrogen · sex-related differences

Alkaline phosphatase (AP; EC 3.1.3.1, orthophosphoric-monoester phosphohydrolase (alkaline optimum)) is present in the human circulation in the form of several isoenzymes, partly because of genetic factors and partly because of post-translational modifications (1). At least three structurally distinct genetic loci code for AP isoenzymes in serum: liver/bone, intestinal, and placental (2). These isoenzymes are easily differentiated by electrophoretic and inhibitory procedures (3), whereas analytical separation of the two main isoenzymes in serum, bone, and liver AP has proved unsuccessful. The small difference between the two lies in the carbohydrate moiety, and is probably the outcome of post-translational modification.

In the method for quantifying bone and liver AP published by Rosalki and Foo (4), they exploited the differential sensitivity of the two isoenzymes to precipitation by wheat-germ agglutinin (WGA), which is a result of the difference in the carbohydrate moiety. However, the method needs to be standardized with regard to both the optimal WGA concentration and the selection of the material containing predominantly liver or bone isoenzymes. I describe here my proposal for standardizing the procedure and present clinical data supporting the usefulness of the method in monitoring AP isoenzymes in postmenopausal women during treatment with estrogens.

Materials and Methods

Measurement of AP isoenzymes. I measured the bone and liver AP isoenzymes according to the method described by Rosalki and Foo (4), mixing 50 μL of serum with 50 μL of WGA solution. The WGA was obtained from two different suppliers: lyophilized WGA (Boehringer Mannheim, Mannheim, F.R.G.), which I reconstituted in distilled water; and WGA in aqueous solution (Kem-en-tec, Copenhagen, Denmark) with 1 mol of NaCl and 15 mmol of NaN₃ per liter. After incubation for 30 min at 37°C, I centrifuged the samples (12,000 × g, 4 min) at ambient temperature and determined the residual AP activity in the supernate as recommended (5). The amount of nonprecipitated AP activity varied with the WGA concentration; also, batches of WGA from the two suppliers, and even WGA batches from the same company (Boehringer Mannheim), had different affinities for the AP in pooled sera from 108 healthy blood donors (Figure 1). I therefore used a single batch of WGA throughout this study (Kem-en-tec) and a WGA concentration (2.5 g/L) that precipitated 50% of the AP activity in the pooled normal serum (Figure 1). The amount of nonprecipitated AP activity was quite different in cord blood, in pooled normal serum, and in serum from a patient with cirrhosis (Figure 2).

Establishing the differential sensitivity of bone and liver AP isoenzymes to precipitation by WGA. To establish the extent of precipitation of bone and liver AP by WGA, I measured total AP activity and nonprecipitated AP activity in serum from 23 samples of cord blood, in serum from 14

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Fig. 1. Nonprecipitated AP activity after addition of various concentrations of three different WGA preparations to pooled serum from blood donors

Broken lines indicate the WGA concentration (2.5 g/L) that precipitated 50% of the AP activity in the pooled normal serum
children (ages nine months to 14 years, admitted mostly because of epilepsy), and in serum from 10 patients (ages 62 to 77 years) with hepatobiliary disease. Because serum from the latter group may contain bilary AP, an abnormal component with a higher molecular mass (5), I pre-incubated 50 μL of these samples at 37 °C for 30 min with 5 μL of a 20 g/L solution of the nonionic detergent Triton X-100, to convert bilary AP to liver AP (4) before addition of WGA. The calculated activities were adjusted for sample dilution. I also investigated blood from 108 healthy donors: 43 women (ages 19 to 55 years) and 65 men (ages 19 to 62 years).

Calculations. The total AP activity of healthy subjects consists of contributions from bone (B), liver (L), and intestinal (I) isoenzymes and may be expressed by the equation:

\[ \text{Total alkaline phosphatase (T) = B + L + I} \]  

(1)

Assuming different sensitivities of the three isoenzymes to precipitation by WGA, a second equation may be expressed as follows:

\[ \text{Nonpptd. acty. (NPA) = (f_B \times B) + (f_L \times L) + (f_I \times I)} \]  

(2)

where \( f_B \), \( f_L \), and \( f_I \) indicate the fraction of AP activity remaining in the supernate after WGA precipitation when only bone, liver, or intestinal alkaline phosphatase is assumed to be present in the sample.

If samples from fasting patients are used, we can disregard the contribution from intestinal alkaline phosphatase, because fasting diminishes this to 0–5% (7).

Two simple expressions can now be derived from equations 1 and 2:

\[ L = \frac{\text{NPA} - (f_B \times T)}{f_L - f_B} \]  

(3)

\[ B = \frac{(f_L \times T) - \text{NPA}}{f_L - f_B} \]  

(4)

I estimated \( f_B \) and \( f_L \) by assaying serum samples containing predominantly liver or bone isoenzymes and serum samples from healthy subjects containing different compositions of bone and liver AP.

Clinical assessment of determination of bone and liver AP isoenzymes. I assessed the method in a double-blind controlled clinical study of treatment with estrogen for prevention of postmenopausal bone loss. I analyzed serum from 20 fasting healthy women in early postmenopause (ages 46–53 years) before and after receiving one and two years of cyclic hormone replacement therapy (n = 9) or no treatment (n = 11). The cycle during the first year was as follows: days 1–24, 3 mg of percutaneous 17β-estradiol, and days 25–28, no hormone. In the second year, treatment on days 13–24 was supplemented with 100 mg of progesterone. Blood was sampled on days 21 to 24 of a cycle and stored at −20 °C until analyzed.

Statistical evaluations. In the estrogen-treated group of women, the change over time was evaluated by use of the paired-sample t-test.

Results

Determination of the values for the factors, \( f_B \) and \( f_L \). Determination of NPA as a percent of total AP activity in the individual specimens, NPA(%), with use of a WGA concentration that precipitated 50% of total AP activity in the pooled normal serum from healthy subjects, gave highly variable results (Figure 2). NPA(%) ranged from 36% to 94% (mean 59%) in healthy women and from 23% to 85% (mean

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Fig. 2. Nonprecipitated AP activity after addition of various concentrations of the same WGA preparation (Kem-en-tec, batch no. 8507) to serum from a patient with liver metastases from a stomach cancer (Ø), to serum from cord blood (Δ), and to a serum pool from healthy blood donors (C).

Inset: precipitated AP activity as a percentage of total AP activity

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Fig. 3. Nonprecipitated AP activity as a percent of total AP activity in serum from cord blood (n = 23), children (n = 14), healthy women (n = 43), healthy men (n = 65), and patients with hepatobiliary diseases (with and without pretreatment with Triton X-100) (n = 10)

Horizontal bars indicate the mean for each group. Arrows on the y-axis indicate the estimated values for the percentage (or fraction) of AP activity remaining in the supernate after WGA precipitation when only bone or liver AP activity is present (\( f_B = 0.00 \) and \( f_L = 0.95 \)).
Table 1. Reference Values for Total AP, Bone-Derived AP, and Liver-Derived AP in Serum

<table>
<thead>
<tr>
<th>Age, y</th>
<th>n</th>
<th>Total Mean (and range)</th>
<th>Bone Mean (and range)</th>
<th>Liver Mean (and range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>2</td>
<td>134 (111–157)</td>
<td>68 (54–81)</td>
<td>67 (57–76)</td>
</tr>
<tr>
<td>20–29</td>
<td>15</td>
<td>133 (99–204)</td>
<td>54 (3–126)</td>
<td>79 (53–120)</td>
</tr>
<tr>
<td>30–39</td>
<td>13</td>
<td>121 (72–192)</td>
<td>46 (7–85)</td>
<td>75 (42–118)</td>
</tr>
<tr>
<td>40–49</td>
<td>10</td>
<td>107 (65–198)</td>
<td>36 (2–80)</td>
<td>71 (27–118)</td>
</tr>
<tr>
<td>50–59</td>
<td>3</td>
<td>135 (129–142)</td>
<td>54 (51–58)</td>
<td>81 (78–84)</td>
</tr>
<tr>
<td>Men</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>4</td>
<td>197 (136–302)</td>
<td>116 (72–167)</td>
<td>81 (36–135)</td>
</tr>
<tr>
<td>20–29</td>
<td>15</td>
<td>189 (127–314)</td>
<td>110 (9–187)</td>
<td>79 (42–127)</td>
</tr>
<tr>
<td>30–39</td>
<td>15</td>
<td>147 (82–181)</td>
<td>80 (50–106)</td>
<td>67 (32–114)</td>
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<tr>
<td>40–49</td>
<td>20</td>
<td>149 (94–287)</td>
<td>67 (10–130)</td>
<td>67 (40–179)</td>
</tr>
<tr>
<td>50–59</td>
<td>7</td>
<td>161 (106–192)</td>
<td>67 (37–91)</td>
<td>95 (69–131)</td>
</tr>
<tr>
<td>60–62</td>
<td>4</td>
<td>153 (108–192)</td>
<td>57 (25–87)</td>
<td>96 (44–143)</td>
</tr>
</tbody>
</table>

47%) in healthy men. The slight predominance of serum from men in the pooled serum used for determination of a suitable WGA concentration explains why the two mean values are not quite balanced around 50%. Newborn babies had a low NPA(%), ranging from 2% to 21% (mean 6%), whereas in children it ranged from 6% to 37% (mean 18%). In patients with hepatobiliary disease with a range of total AP activity from 655 to 2942 U/L (mean 1183 U/L), NPA% was high, ranging from 53% to 77% (mean 67%). This was increased by pretreatment with Triton X-100 to a range from 67% to 91% (mean 80%). On the measurements described above, the values for f1 and f2 were determined to be 0.95 and 0.00, respectively. When the values for f1 and f2 have been found and the total AP activity and NPA are known, it is possible to calculate bone and liver AP isoenzymes in each individual specimen by means of expressions (3) and (4).

Table 1 gives the means and the ranges for total and bone- and liver-derived AP activity for both women and men. Whereas the liver AP activity is almost identical in women and men, the bone AP activity is about 70% higher in men than in women. In patients with hepatobiliary disease, bone and liver AP activities ranged from 49 to 315 U/L (mean 172 U/L) and 590 to 2735 U/L (mean 1011 U/L), respectively.

Accuracy. Serum from a child and a patient with hepatobiliary disease were mixed in various ratios, of which 50 µL was pre-incubated with 5 µL of 20 mL/L Triton X-100 solution. The method was then performed as previously described. The results demonstrated an acceptable agreement between the found and the expected values for total, bone, and liver AP activity.

Precision. To evaluate the interassay and intra-assay variation, I used pooled sera from healthy subjects and from cord blood. The results are shown in Table 2. The interassay variation for bone and liver AP activities in serum ranged from 9% to 14%.

Clinical evaluation of determination of bone and liver isoenzymes in serum. The group of nine women who were receiving cyclic hormone treatment showed, after one year's treatment with estradiol, a significant (P <0.01) decrease in total AP activity. This was entirely ascribable to a decrease
in bone AP activity, because the liver-derived AP activity was unchanged during this period (Figure 4). The other group receiving no treatment had a slight but insignificant increase in the mean value for total AP activity, whereas the increase in bone AP activity was significant (P < 0.05). Liver AP activity was unchanged.

The meaning of the chosen values for \( f_0 \) and \( f_1 \) in these findings was investigated by applying other probable values for \( f_0 \) and \( f_1 \) in the group of women receiving hormone treatment. Three sets of values were tried: 0.05/0.95, 0.05/0.85, and 0.00/0.85. Only small parallel displacements of the level, within two times the SEM, were observed. If \( f_0 \) was increased from 0.00 to 0.05, the bone-derived AP activity increased and the liver AP activity decreased, whereas if \( f_1 \) was decreased from 0.95 to 0.85, liver AP activity increased and bone AP activity decreased.

**Discussion**

The concentration of WGA to be used in the determination of bone and liver AP activity should be carefully adjusted if one is to best exploit the different reactivities of the lectin for bone and liver AP activity. A concentration that precipitated 50% of the AP activity of pooled normal serum was preferred, because this concentration gave a clearcut difference between precipitated bone-derived and liver-derived isoenzyme activity.

AP isoenzymes prepared from organ sources may behave differently from their corresponding isoenzymes in serum (4). The use of serum standards instead of organ-derived standards can overcome this problem, but probably there are no serum samples that contain only one AP isoenzyme. However, because the liver function in newborn babies is still immature, the AP in cord blood is almost all of skeletal origin and is thus highly sensitive to WGA. In several cord-blood samples, the residual activity after WGA addition was close to the lowest detectable, about 5–10 U/L, which justifies the choice for an \( f_0 \) value of 0.00. Conversely, most of the AP activity in sera from patients with hepatobiliary disease was left in the supernate by WGA—on average 80% after addition of Triton X-100. I expected a higher proportion of precipitated AP activity to be left, because that amount means that bone AP activity is, on average, two to three times greater than in healthy subjects. One explanation might be that biliary AP, known to be present in half the patients with hepatobiliary disease (8), might be precipitated by WGA, and pretreatment of the samples with Triton X-100 should obviate this effect (4) by converting it to liver AP. However, biliary AP cannot be entirely converted to normal liver AP by Triton X-100 when evaluated by electrophoresis (6, 8). Another possibility might be that bone AP activity is in fact increased. A value of 0.95 for \( f_1 \) was chosen; lower values such as 0.85 or 0.90 would imply only small changes of bone and liver AP activities in patients with osteoporosis as compared with an \( f_1 \) value of 0.95 and would imply negative values for bone AP activity in a few healthy subjects.

The duration of and temperature for incubation of samples and WGA were observed as originally described (4). The precipitation, however, seems to take place promptly, and thus it is rather insensitive to changes in time and temperature, although this has not been investigated systematically.

In the serum of healthy adults, liver AP activity is essentially the same in the two sexes (9, 10, 11) when estimated by heat inactivation or the WGA method demon-
Sensitive, Rapid Procedure for Time-Resolved Immunofluorometry of Lutropin
Mohammad J. Khoeravil, Robert C. Morton, and Eletherios P. Diamandis

In this new immunofluorometric method for quantification of lutropin in serum, the "sandwich" principle is combined with time-resolved fluorescence measurements, with the europium chelate 4,7-bis(chlorosulfofeny)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) used as label. A monoclonal antibody to the alpha-subunit of lutropin is adsorbed onto the walls of white-opaque microtiter wells to form the solid-phase capture antibody, and a biotin-labeled soluble monoclonal antibody is used for antigen quantification. The detection system is completed with streptavidin, which has been linked to a protein bulking agent labeled with multiple BCPDA residues. In the presence of excess europium, the fluorescence of the final complex attached to captured lutropin molecules is measured on the dried solid phase with an automated time-resolved fluorometer. The assay can be performed as a rapid (<60 min incubation) or regular (150 min incubation) procedure. The rapid assay is well-suited for routine daily monitoring of increasing or ovulatory lutropin concentrations; the regular assay, with its greater sensitivity (0.5 int. unit/L), is a practical procedure for lutropin measurements in hyposecretory states. The assay measures up to 240 int. units/L, and results compare well with those by a commercially available radioimmunoassay, an immunoradiometric assay, and another time-resolved immunofluorometric procedure.

Additional Keyphrases: RIA, immunoradiometric assay compared with hypersecretory states • fertility studies

Measurement of lutropin (luteinizing hormone, LH) in human serum is useful in many areas of reproductive endocrinology (1–7). The radioimmunoassays (RIAs) used routinely for many years (8–11) are now being replaced with the more sensitive immunoradiometric procedures (12–13). A number of non-isotopic detection systems have recently been introduced in which enzymes (14), erythrocytes (15), or fluorescence probes (16) are used as labels.

The fluorescent Eu³⁺ complexes exhibit attractive properties as immunological tracers (17–19). Because the fluorescence emitted from these complexes is long lived, the tracer can be detected after a pulsed excitation with a gated fluorometer working in a time-resolved mode. Other favorable features of the Eu³⁺ complexes have been reviewed (19–21). We have recently described a time-resolved immunofluorometric assay for chorionic gonadotropin with a new europium chelate [4,7-bis(chlorosulfofeny)-1,10-phenanthroline-2,9-decarboxylic acid, BCPDA] as label (20). Using this same chelate, we have developed an immunofluorometric assay of lutropin. It is a two-site "sandwich"-type assay in which a monoclonal antibody to lutropin-alpha subunit, immobilized in microtiter wells, forms the solid phase, while a biotin-labeled monoclonal antibody to lutropin-beta subunit forms the detection antibody. The detection system is completed by interfacing the biotinylated antibody to streptavidin, which has been covalently linked to a bulking protein agent carrying multiple BCPDA residues. In the presence of excess europium, the final fluorescent complex on the dried solid phase is then measured in an automated time-resolved fluorometer.

Contamination with europium constitutes the principal drawback of the currently available time-resolved fluorometric immunoassays described by a number of investigators for lutropin (16) and other analytes (21). In the present procedure, this major disadvantage has been eliminated by using BCPDA as label and performing the assay in the presence of a saturating concentration of europium.

Materials and Methods

Instrumentation

Time-resolved fluorescence measurements at the bottom of dried white microtitration wells were performed with the Model 615 Immunoanalyzer (CyberFluor Inc.). Data reduction was done automatically by the machine (19). Time-resolved fluorescence measurements of liquids (for the "Del-fia" kit) were performed with the Arcus fluorometer (LKB Wallac, Turku, Finland). Radioactivity counting was performed with the LKB 1275 Minigamma counter.

Materials

Chemicals. Human follitropin (follicle-stimulating hormone) was from Scripps Labs., San Diego, CA 92103. Human choriongonadotropin was from Calbiochem–Behring Diagnostics, La Jolla, CA 92037. Human thyrotropin (thyroid-stimulating hormone) was from Sigma Chemical Co., St. Louis, MO 63178. Bovine serum albumin, bovine globulin, bovine thyroglobulin, and streptavidin were also from Sigma. Sulfoaceticinimidyl 6-(biotinamido) hexanoate (NHSC-LC-Biotin) was from Pierce Chemical Co., Rockford, IL 61105. Europium(III) chloride hexahydrate was from Aldrich Chemical Co., Milwaukee, WI 53233. White opaque microtiter strips (12-well), "Microfluor," are products of CyberFluor Inc., 179 John Street, Toronto, Ontario, M5T 1X4, Canada.

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