Alkaline Phosphatase Isoenzymes and Osteocalcin in Serum of Normal Subjects*†

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ABSTRACT

Clinical laboratory tests are increasingly being used to evaluate individuals for osteoporosis and other metabolic bone diseases. Serum bone alkaline phosphatase (AP) [EC 3.1.3.1, orthophosphoric-monoester phosphohydrolase (alkaline optimum)] and osteocalcin are used to assess osteoblastic activity. Although methods for assessing relative amounts of AP isoenzymes continuously appear in the literature, no single method is satisfactory for quantification. Polyacrylamide gel electrophoresis with densitometric scanning combined with two-point heat inactivation was used to obtain quantitative values for AP isoenzymes. Serum bone AP concentrations correlated positively and significantly with serum osteocalcin concentrations obtained by radioimmunoassay for women. Men had significantly higher total alkaline phosphatase and bone AP than women, whereas liver AP concentrations did not differ between the two groups. Bone AP correlated negatively and significantly with age in men, but not women. Osteocalcin concentrations tended to be higher in men, but not significantly.

Introduction

Bone alkaline phosphatase (AP) and osteocalcin in serum reflect osteoblastic activity and, with urinary hydroxyproline, bone turnover rates. These analytes may serve as diagnostic or predictive markers for metabolic bone diseases such as osteoporosis.

Although AP measurement is one of the most frequently performed clinical laboratory tests, and quantification of AP isoenzymes is clinically useful in a significant proportion of cases in which alkaline phosphatase is requested, there is no single, satisfactory method for quantification of AP isoenzymes. Electrophoresis has been the method of choice for qualitatively identifying multiple isoen-
zymes, and various inhibition methods have been used to quantify isoenzymes. A novel combination of methods was used to quantify AP isoenzymes in a healthy population and to determine its association with osteocalcin for use in studies assessing osteoblastic activity.

Materials and Methods

Serum was obtained from 36 women, 31 men, and one woman in the third trimester of pregnancy, all of whom fasted for eight hours. Subjects were noninstitutionalized, nonmedicated individuals aged 27 to 66 years.

Kits were obtained* between April 1985 and July 1986, and five lot numbers were used. The kits contained 7.7 percent polyacrylamide gels and 5-bromo-4-chloro-3-indolylphosphatase p-toluidine salt substrate/staining solution, 21.0 mg per dL in buffer. Buffer was Tris 66.1 percent w/w, boric acid 33.9 percent w/w at pH 8.4 ± 0.2. Acrylamide gels of 7.7 percent were made with three percent stacking gels, with and without wheat germ agglutinin (WGA),† 400 mg per L, final concentration, and compared with commercial gels.

Twenty five μL of serum were applied to gels, and three mA per tube were applied for approximately 80 minutes or until the albumin, a visible, slightly yellow band, reached the bottom of the gel. Gels were then placed in the substrate/stain solution and allowed to stain for two hours. Gels were washed with and stored in, seven percent acetic acid and scanned with a densitometer‡ adapted for tube gels by attaching a mylar backing from an agarose electrophoresis gel to the scanning stage. When bone and liver peaks were not completely resolved, electrophoretic scans of heat-inactivated aliquots were used to determine where to place cuts to integrate and quantify peaks.

Four hundred microliters of serum were placed in 12- × 75-mm Pyrex tubes in a 56°C circulating water bath, removed after 10 and 15 or 15 and 25 minutes to an ice bath, and allowed to cool for five minutes. Electrophoresis and quantification of total AP activity was then performed. Liver AP was also calculated by using activities or log activities of total alkaline phosphatase (TAP) at 10 and 15 or 15 and 25 minutes and extrapolating to zero time.

Alkaline phosphatase activity was measured at 37°C on the Cobas Bio centrifugal analyzer by using the Cobas Bio optimized method and reagents§ on untreated and heat-inactivated serum. Osteocalcin was measured using a double antibody radioimmunoassay.¶

T-tests were used for equality of means and P-values of 0.05 and less were considered to indicate significance.

Frequency distributions were analyzed for normality using Kolomogrov's D-statistic for goodness of fit. Distributions that were not normal were appropriately transformed. The significance levels for correlation coefficients were determined by using the F-statistic.

Selected samples with intestinal bands and a sample from a third-trimester pregnancy were heated for 30 minutes at 60°C to identify placental bands and distinguish them from intestinal bands.

Results and Discussion

Sixty-seven normal sera and one from a third-trimester pregnancy were examined by the technique described. In figure 1 are shown the electrophoretic pat-

* Alkphor System 100 kits, California Immuno Diagnostics, San Marcos, CA 92069.
† Sigma Chemical Co., St. Louis, MO 63178.
‡ Beckman Appraise Densitometer, Beckman Instruments, Fullerton, CA 92634.
§ Roche Diagnostic Systems, Nutley, NJ 07110.
¶ Immuno Nuclear Corp., Stillwater, MN 55082.
SERUM ALKALINE PHOSPHATASE AND OSTEOCALCIN

Figure 1. Polyacrylamide gel electrophoresis (PAGE) of alkaline phosphatase (AP) isoenzymes. A. Human bone AP heated at 56°C for 0, 3, and 6 minutes (first three tubes) and liver AP heated at 56°C for 0, 3, and 6 minutes (last three tubes). B. Placental AP heated at 56°C for 0, 3, and 6 minutes (first three tubes) and serum AP heated at 56°C for 0, 3, and 6 minutes (last three tubes).

Mean coefficients of variation (CVs) for total alkaline phosphatase (TAP) from duplicate determinations on heated (56°C for 15 min) and unheated aliquots were 8.6 ± 7.0 percent and 3.9 ± 4.2 percent respectively (n = 7). Between-run precision figures for TAP on a control serum heated at 56°C for zero, ten, and 15 minutes were 1.4 percent, 8 percent, and 9.7 percent, respectively (n = 26 for each time).

The 99 percent confidence interval for TAP activity in men and women ages 27 to 66 years was 19.5 to 112.7 U per L (n = 67), with a range of 20 to 122 U per L.
TABLE I
Alkaline Phosphatase Activities Shown in 67 Adults*

<table>
<thead>
<tr>
<th></th>
<th>TAP</th>
<th>Bone</th>
<th>Liver</th>
<th>Intestinal</th>
<th>Osteocalcin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men</td>
<td>72.4 ± 19.9†</td>
<td>29.9 ± 11.3</td>
<td>33.0 ± 12.0</td>
<td>10.9 ± 5.7</td>
<td>3.9 ± 2.2‡</td>
</tr>
<tr>
<td></td>
<td>(n=31)</td>
<td>(n=31)</td>
<td>(n=31)</td>
<td>(n=13)</td>
<td>(n=22)</td>
</tr>
<tr>
<td>Women</td>
<td>60.7 ± 18.2</td>
<td>23.7 ± 11.0</td>
<td>30.4 ± 12.2</td>
<td>10.3 ± 7.3</td>
<td>3.3 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>(n=36)</td>
<td>(n=36)</td>
<td>(n=36)</td>
<td>(n=15)</td>
<td>(n=28)</td>
</tr>
<tr>
<td>All</td>
<td>66.1 ± 19.5</td>
<td>26.5 ± 12.7</td>
<td>31.5 ± 12.7</td>
<td>10.6 ± 6.5</td>
<td>3.6 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>(n=67)</td>
<td>(n=67)</td>
<td>(n=67)</td>
<td>(n=28)</td>
<td>(n=50)</td>
</tr>
</tbody>
</table>

P < 0.01§ P < 0.025 N.S. N.S. N.S.

Serum concentrations of TAP, AP isoenzymes, and osteocalcin in men and women are given in table I. Values obtained here for TAP and isoenzymes are similar to those previously reported.26,32

About equal activities were found for bone and liver in this population, a finding consistent with those of previous studies.9,26,37 Men had a higher mean TAP, p < 0.01, primarily owing to higher mean bone AP, p < 0.025. These findings agree with previous reports that men have higher TAP than women for subjects younger than 50 years because of bone AP.14,32,37 It was observed that subjects older than 50 have higher mean TAP than those younger than 50, but differences observed here were not statistically significant, possibly because of the small sample of those over 50. Mean ages for men and women, 40 ± 8 (n = 31) and 43 ± 8 (n = 36), respectively, were not significantly different.

Mean activities of TAP and isoenzymes calculated from electrophoretic peaks before and after heating are given in table IIA, and residual activities after heating for 10 and 15 minutes are given in table IIB. Although residual activity of isoenzymes after heating at 10 and 15 minutes did not differ between men and women (table IIA), the percentage of residual activity was higher in women than in men for bone and liver isoenzymes (figure 2B). The 99 percent confidence interval for bone at 15 minutes, 0 to 18 U per L, is virtually distinct from the 99 percent confidence interval for liver at 15 minutes, 18 to 54 U per L. However, it was not possible in our laboratory to determine the predominating isoenzyme by the percentage of TAP left after 10 and 15 minutes, as others have suggested.

Theoretically, if serum is heated at 56°C long enough to eliminate bone isoenzyme, subsequent points could be extrapolated to zero to find the total activity of all isoenzymes present other than bone. Others have found that residual activities from specimens heated for 15 and 25 minutes at 56°C are sufficiently free of bone isoenzyme to do this.25 Because zero percent to 16 percent residual activity of bone at 15 min-

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†Mean U/L ± 1 S.D.
‡Mean ng/mL ± 1 S.D.
§Significance of differences between males and females
TAP = total alkaline phosphatase
N.S. = not significant
TABLE II
Heat Stability of Alkaline Phosphatase Isoenzymes at 56°C

A. Absolute Activity in U/L Before and After Heating at 56°C

<table>
<thead>
<tr>
<th></th>
<th>Total Alkaline Phosphatase</th>
<th>Bone</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>0 min</td>
<td>10 min</td>
</tr>
<tr>
<td>Men</td>
<td>36</td>
<td>72.4±19.9*</td>
<td>21.5±7.0</td>
</tr>
<tr>
<td>Women</td>
<td>31</td>
<td>60.7±18.2</td>
<td>24.0±7.0</td>
</tr>
<tr>
<td>All</td>
<td>67</td>
<td>66.1±19.5</td>
<td>22.6±7.2</td>
</tr>
</tbody>
</table>

P < 0.01† N.S. N.S. P < 0.025 N.S. N.S. N.S. N.S. N.S.

P < 0.05‡ N.S. N.S. P < 0.025 N.S. N.S. N.S. N.S. N.S.

B. Percent Activity Remaining after Heating at 56°C

<table>
<thead>
<tr>
<th></th>
<th>Total Alkaline Phosphatase</th>
<th>Bone</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>10 min</td>
<td>15 min</td>
</tr>
<tr>
<td>Men</td>
<td>31</td>
<td>33.6±8.1‡</td>
<td>22.3±6.7</td>
</tr>
<tr>
<td>Women</td>
<td>36</td>
<td>35.6±5.1</td>
<td>24.3±4.3</td>
</tr>
<tr>
<td>All</td>
<td>67</td>
<td>34.6±6.6</td>
<td>23.4±5.6</td>
</tr>
</tbody>
</table>

P < 0.025 N.S. N.S. P < 0.025 N.S. N.S. N.S. N.S. N.S.

Mean ± 1 S.D.
†Significance of differences between males and females
‡Mean percent residual activity ± 1 S.D.
N.S. = not significant

utes with bone activity persisting for 25 minutes was seen, and because heating at 15 and 25 minutes did not result in a closer correlation between activities obtained by electrophoresis and heat inactivation, 10 and 15 minutes were used for convenience. In the final method described here, heat inactivation was used only to reveal unresolved peaks not to extrapolate to zero time for activity other than bone.

When the log of AP activities was used after heat inactivation for 10 and 15 minutes to extrapolate to zero time to determine total liver AP activity, values were obtained that correlated with liver values obtained from electrophoretic peaks, R = 0.7220, p < 0.0001 (figure 3). The CV was higher (16 percent) with extrapolation to zero than with electrophoretic peaks (6.6 percent). Extrapolation to zero time to determine total liver AP was only appropriate in 61 percent of the cases in which no intestinal AP was present.

Bone AP is selectively precipitated by wheat germ agglutinin (WGA) because of the presence of glycoprotein side-chains. A sharper bone band on polyacrylamide gel electrophoresis (PAGE), as reported for agarose and cellulose acetate electrophoresis, was attempted to be obtained by incorporating WGA into acrylamide gels. Although gels made "in house" without WGA gave results identical to commercial gels, adding WGA did not enhance isoenzymes separation. Rather, spreading of the liver band occurred in lectin containing gels. Rosalki and Ying Foo found minimal precipitation of liver isoenzymes as well as bone. Our electrophoretic patterns indicated that precipitation of liver enzymes was sufficient to interfere with resolution.

Despite agreement that intestinal AP is present in sera of normal subjects, the prevalence and amount have not been established. A band was found compatible with the electrophoretic mobility and heat stability of intestinal AP with a mean activity of 10.6 ± 6.5 U per L in 39 percent of fasting subjects, a significantly higher value than the 25
percent observed by Moss\textsuperscript{24} or the 10 percent found by Schiele et al\textsuperscript{32} using \textit{L}-bromo-tetramisole inhibition. The prevalence of intestinal AP cannot be determined until more data are accumulated. Heating at 56°C for 30 minutes virtually eliminated intestinal bands but did not destroy the activity of placental AP.

A statistically significant correlation was not found between TAP or liver AP and age in subjects older than 23 years. Bone AP correlated negatively and significantly with age in men, $r = 0.3816, p = 0.0341 (n = 31)$ (figure 4) but not in women. Although the increase in activity of TAP during childhood and adolescence, primarily owing to bone isoenzyme, is well established\textsuperscript{4,10,31} the present authors agree with Schiele et al\textsuperscript{32} that TAP does not vary with age from 20 to 50 years. The mean TAP for subjects from 25 to 50 years old was found to be lower than that for subjects over 50 years, but differences were not statisti-
cally significant, perhaps because of insufficient numbers of subjects older than 50 years.

Osteocalcin is a noncollagenous protein (MW 6,000) that binds calcium to residues of the amino acid gamma-carboxyglutamic acid. The gamma-carboxyglutamic acid relies on vitamin K for its synthesis. Osteocalcin makes up more than 20 percent of the noncollagenous protein of bone. Nanomolar concentrations of osteocalcin are found in serum and are thought to reflect metabolic states of bone.

The Immunonuclear radioimmunoassay kit was used to determine osteocalcin concentrations. This method was compared to a similar, well-characterized radioimmunoassay, and although values obtained with the kit were lower than those obtained with the reference method, the results correlated well (r = 0.979).

Osteocalcin concentrations tended to be higher in men than women, 3.9 ± 2.2 and 3.3 ± 2.1 ng per mL, respectively, but differences were not statistically significant. The 99 percent confidence limits for osteocalcin were 0 to 8.9 ng per mL. Bone AP and serum osteocalcin correlated positively and significantly in women, r = 0.5768, P < 0.0013 (n = 28) but not in men (figure 5). Osteocalcin was not significantly correlated with TAP or liver AP. Concentrations have been reported to decrease, increase, or not change with age. Our findings agree with those of Catherwood et al that osteocalcin does not correlate with age in adults.

A method for quantifying AP isoenzymes to measure the bone isoenzyme for osteoblastic activity has been presented. Reference limits for AP isoenzymes and serum osteocalcin have also been presented.

Differentiating and quantifying AP isoenzymes are useful procedures for following the development of bone or liver metastases, identifying bone involvement in liver disease and in renal failure, and identifying elevated isoenzymes when total activity is normal. Moss estimates that quantitative analysis of isoenzymes would be useful in 40 percent cases in which AP is requested.

Alkaline phosphatase isoenzymes have been quantified by electrophoretic methods based on charge differences and methods relying on differences in resistance to inhibitors such as heat, phenylalanine, urea, lectin containing gels, and immunological methods. Quantitative heat inactivation

**Figure 5. Comparison of serum osteocalcin and bone alkaline phosphatase (AP) activity in women.**
methods that rely on multiple point determinations do not account for isoenzymes which have heat stability similar to liver such as intestinal or placental-like isoenzymes. Inactivation constants used for bone and liver isoenzymes neglect differences in heat stability between sera, probably because of such factors as pH.

The method presented here is similar to that of Epstein et al with the exception that densitometric scanning is used to quantify isoenzymes. In this method heat inactivation is used only to determine the position of incompletely resolved peaks on PAGE.

When values were compared for liver isoenzyme activity obtained with a two-point heat inactivation with extrapolation of zero time for integration of electrophoretic peaks, results were similar; however, the electrophoretic method combined with heat inactivation offered the advantages of better precision and identification of isoenzymes other than liver and bone. Bone AP, by this method, correlates with osteocalcin in the normal range.

References


