Alkaline Phosphatase Isoenzymes in Serum Determined by High-Performance Anion-Exchange Liquid Chromatography with Detection by Enzyme Reaction

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This rapid, reproducible method for separating and determining individual alkaline phosphatase (EC 3.1.3.1) isoenzymes in serum is based on high-performance liquid chromatography with a weak anion-exchange column (SynChropak AX 300). The isoenzymes so resolved are detected by using an on-line enzyme reaction followed by spectrophotometric monitoring at 405 nm of the 4-nitrophenol formed. Complete diagnostic profiles of the various isoenzymes present in normal and pathological sera are obtained within 20 min. The mean (and SD) normal concentrations of the bone B1 and intestinal isoenzymes in serum of adults were 3.7 (4.3) and 4.5 (3.9) U/L, respectively (n = 14), and of the bone isoenzyme B2 and liver isoenzymes L1 and L2, 5.8 (8.6), 33.0 (10.6), and 12.0 (4.8) U/L, respectively (n = 17). Concentrations of the B2 and L1 isoenzymes in adults over age 40 years differed significantly from those in adults younger than 40 years, that of bone isoenzyme being lower (P <0.05) and that of the liver isoenzyme being higher (P <0.001) in the younger adults.

Additional Keyphrases: age-related differences · chromatography · anion-exchange · reference values · bone disease · Paget's disease

Alkaline phosphatase (AP; orthophosphoric-monoester phosphohydrolase [alkaline optimum; EC 3.1.3.1]) is a heterogeneous zinc-containing dimeric glycoprotein encoded by at least three gene loci (1). Two AP isoenzymes—intestinal and placental—are tissue-specific; a third isoenzyme is a tissue-nonspecific AP isoenzyme found mainly in bone, liver, and kidney (1). However, the tissue-nonspecific APs differ by post-translational modifications—that is, in their carbohydrate content.

The various AP isoenzymes can be distinguished by using different stereospecific noncompetitive inhibitors (2), heat inactivation (3), immunological methods (2, 4), and electrophoresis (5, 6), or some combination of these methods. However, these methods can only partly separate or give only a semiquantitative estimation of the different isoenzyme fractions.

High-performance liquid chromatography (HPLC), a powerful method for separating proteins (7), has recently been applied to the separation of the various isoenzymes of lactate dehydrogenase and creatine kinase (8). Schönau et al. (9, 10) described an HPLC method for separating AP isoenzymes by using a strong anion-exchange column (Mono Q HR 5/5). They separated several AP isoenzymes originat-

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HPLC system. We used two Model 6000A solvent-delivery systems pumps, with a Model 720 system controller, a Model 450 variable-wavelength detector, and a WISP (Waters Intelligent Sample Processor) 710A, all from Waters Associates, Harrow, U.K.; or a Model 7125 manual injector (Rhodyne, Cotati, CA) and a RE 541.20 potentiometric recorder (Venture, London, U.K.). The column effluent from a 25 cm × 4.2 mm (i.d.) SynChropak AX 300 column (Anachem, Luton, U.K.) was mixed in a tee-junction (Waters, P/N 01-0165) with the AP substrate stream pumped by a third Model 6000A pump. The mixture was directed through an incubation coil at 25 °C (Waters P/N 26804 stainless-steel tubing, 600 cm × 0.51 mm) to the detector set at 405 nm. A Waters pre-column filter (P/N 84560) was used to protect the HPLC column.

Reagents. We used chemicals from Sigma Co., Poole, U.K. All HPLC reagents and buffers were made up in glass-distilled water, degassed, and filtered through a 0.45-μm (pore size) filter (Millipore, Molsheim, France). The AP substrate was 4-nitrophenyl phosphate, 1.76 mmol/L, in 0.1 mol/L diethanolamine hydrochloride (pH 10.4) containing 2.0 mmol of MgCl2 and 5 mL of "Non-Ionic Wetting Agent" (Technicon Instruments Corp., Tarrytown, NY) per liter. HPLC buffer A was 20 mmol/L Tris acetate (pH 7.9); buffer B was 20 mmol/L Tris acetate (pH 7.9) containing 0.5 mol of sodium acetate per liter. Human placental AP was obtained from Sigma. For comparison, we also obtained a specimen of small intestine from a patient undergoing intestinal surgery. The intestinal sample (~5 g) was washed with phosphate-buffered isotonic saline, homogenized, and centrifuged. We were not able to observe any isoenzyme activity other than the intestinal AP in this supernatant preparation.

Serum samples. Sera from a group of healthy adults (n = 17), adult patients with Paget's disease (n = 7), liver disease (cholestasis and hepatitis, n = 5), or bone disease (metastasis of bone, osteomalacia, and osteoporosis, n = 4) were stored at 4 °C and analyzed within two weeks. Before analysis, we diluted the samples from two- to 10-fold in isotonic saline (0.15 mol/L NaCl), depending on the total activity of AP.

Total AP activity. AP in serum was measured with a Technicon SMAC II continuous-flow analyzer, in which the serum sample is added to a stream of 4-nitrophenyl phosphate and incubated for a fixed time at pH 9.9 and 37 °C. The liberated 4-nitrophenol is separated from interfering substances by dialysis, and the absorbance measured at 410
nm is compared with that derived from a sample of known enzyme activity. One unit (U) of AP catalyzes the reaction of one micromole of substrate per minute under the given conditions. Our reference interval for AP activity in serum from adults is 35–105 U/L. We used this total AP activity value after area integration of the peaks obtained by HPLC to calculate the respective isoenzyme values.

**Heat inactivation.** For differential heat inactivation we heated the samples for 30 min at 56 °C, then quickly cooled them under running water.

**HPLC procedure.** We injected a 100-μL sample into the chromatograph, and separated the isoenzymes by a 15-min linear gradient from the initial condition of 13% (by volume) of buffer B in buffer A to 97% of B in A, holding at that composition for 10 min, and then returning to the initial conditions via a 5-min linear gradient. The flow-rate was 1.0 mL/min for the separation, and 1.2 mL/min for the substrate pump. Under these conditions, the column had a back pressure of about 3.5 MPa. Alternatively, for even faster screening of the various isoenzymes present in the serum samples, a faster gradient of 12 min, starting with 17% of B in A, can be used.

**Statistical methods.** We used logarithmic transformation to normalize skewed data for statistical comparisons (Student's t-test) or for calculations of the reference ranges. Means and standard deviations were calculated from the nontransformed data.

**Results**

The enzyme reaction detection assay for the AP isoenzymes separated by HPLC allowed a reaction time of ~0.5 min in the flow-through incubation coil. The constant pH of the combined AP substrate and sample stream was 9.9, which is optimal for the activity of AP in vitro (1). The HPLC column was stable for at least 500 injections; after ~300 injections we performed an extensive wash as specified by the manufacturer.

We established the linearity of the method by using the isoenzyme preparations and different pathological sera (not shown) over the range of 0–330 U/L for each of the isoenzymes. We could account for 93.2–95.9% of the respective AP isoenzyme under differential elutions of either enzyme preparations or pathological sera were added to a normal serum. For routine use the serum samples were diluted to yield a total AP activity in the range of 30 to 330 U/L, from which a 100-μL aliquot was injected. The detection limit was 0.5 U/L for the intestinal and B1 isoenzymes, and 2 U/L for the B2, L1, L2, and L3 isoenzymes (peak >2 times the baseline noise at the detector setting of 0.05 A). The between-assay CV, determined by a repeated analysis of the same sera (n = 10) on consecutive days, was <5.1% for each of the AP isoenzymes.

Under the assay conditions described, as many as four distinct AP isoenzyme peaks were observed in serum from normal adults (Figure 1). The first of these peaks contained both intestinal and an early-eluting bone isoenzyme (hereinafter called B1). The other peaks were, in order of elution, bone isoenzyme B2, liver isoenzymes L1, L2, and L3. The last was not detected in the normal adults. No placental isoenzyme was detected, because none of the subjects was pregnant. The individual quantification of the intestinal and B1 isoenzymes was achieved by differential heat inactivation and by subsequent HPLC analysis (Figures 1 and 2), which resulted in almost complete removal of the AP activity originating from bone, the other AP isoenzymes being more heat stable. We then calculated the intestinal and B1 isoenzyme activities, using similar principles as previously described by us for amylase isoenzymes (12).

Table 1 shows the heat inactivation and elution characteristics of the various isoenzymes.

Mean (and SD) total alkaline phosphatase activity in the serum of the normal adults was 58.7 (12.9) U/L (n = 17, range 32–87 U/L). Results for their AP isoenzymes are presented in Table 2. In the case of the bone isoenzymes B1 and B2, the distribution of the activities was skewed to the left, but approximated a gaussian distribution after log transformation. The younger adults had significantly lower concentrations of bone isoenzyme B2 (P <0.05) and higher concentrations of liver isoenzyme L1 (P <0.001) compared with those in adults over 40 years of age. No sex-related differences were observed.

Characteristic and diagnostic isoenzyme patterns were obtained in patients with different types of liver and (or) bone diseases, as is illustrated in Figure 2. We analyzed sera from seven patients with Paget's disease having a total mean AP activity of 194.3 (SD 21.2, range 112–353) U/L. The combined bone B1 and intestinal AP isoenzyme activity was 35.1 (SD 22.3) U/L, and those of B2, L1, and L2 were 93.2 (65.3), 57.9 (29.5), and 6.8 (14.6) U/L. The bone B2 AP isoenzyme activity in these patients was highly significantly greater (P <0.001) than that in the normal individuals. In one patient with Paget's disease, the isoenzyme was within the normal range (18.9 U/L), whereas L1 was increased (84.1 U/L) and the activity concentration of the pathological liver isoenzyme L3 was 7.7 U/L.

**Fig. 1.** Chromatographic profiles of normal serum samples analyzed as described in the text

Abbreviations for the various AP isoenzymes and the relative retention times are given in Figure 1A. C, total AP activities were 54, 72, and 59 U/L, respectively. D, same as C, but after heat inactivation
Discussion

In relatively few studies have the different serum AP isoenzymes been sufficiently resolved to define the reference intervals for normal subjects. The main problem with the various electrophoretic and inhibition methods is insufficient separation of the activities of the bone and liver isozyme (2, 3, 5, 6). An identical problem with the immunological methods thus far reported (2, 4) has given rise to conflicting data concerning the relative activities of these fractions. Thus the proportion of liver AP isozyme activity is variously reported as greater than (10), equal to (13), or less than (14) that of the bone AP isozyme in normal adults. The present separation method overcomes these problems of resolution in most cases. Our results, which are in general agreement with the findings of Schönau et al. (10), indicate that the activity of the bone isozyme ranged from less than 5% to 98% (mean 21%) of that of the liver AP isozyme. This is considerably less than that recently proposed by Kuwana et al. (14). Our results for the intestinal AP isozyme activity (0–18.9 U/L) agree well with results of Mulivor et al. (2) and Bailyes et al. (15), who used a highly specific monoclonal antibody capture assay.

Table 1. Differential Heat Inactivation of Various AP Isoenzymes, Their Retention Times (tᵣ), and Relative Retention Time *

<table>
<thead>
<tr>
<th>AP Isoenzyme</th>
<th>Remaining (%)</th>
<th>tᵣ min</th>
<th>Relative Retention Time</th>
</tr>
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<tbody>
<tr>
<td>Bone B1</td>
<td>5</td>
<td>4.8</td>
<td>0.52</td>
</tr>
<tr>
<td>Bone B2</td>
<td>6</td>
<td>9.2</td>
<td>1.00</td>
</tr>
<tr>
<td>Liver L1</td>
<td>84</td>
<td>11.1</td>
<td>1.21</td>
</tr>
<tr>
<td>Liver L2</td>
<td>76</td>
<td>13.3</td>
<td>1.45</td>
</tr>
<tr>
<td>Liver L3</td>
<td>82</td>
<td>15.3</td>
<td>1.66</td>
</tr>
<tr>
<td>Intestinal</td>
<td>44</td>
<td>4.8</td>
<td>0.52</td>
</tr>
<tr>
<td>Placental</td>
<td>68</td>
<td>10.0</td>
<td>1.09</td>
</tr>
</tbody>
</table>

*After heat inactivation at 56 °C for 30 min. Relative to AP isozyme B2.

Whereas those divergent results in various studies may be explained by methodological differences, differences in race, age, and sex selection of the reference material may also be important. The proportions of the bone B2 and liver L1 isoenzymes differed according to subject's age in our study. Further data are required to investigate this possibility.

The present method is sensitive enough to quantify the low activities of the AP isoenzymes in serum in healthy persons, and completely diagnostic isoenzyme profiles were obtained in all the patients we studied. The biliary isoenzyme (L3) was present in cholestasis, and it is a diagnostic marker of the disease (9, 10). Increases in the other liver AP isoenzymes, L1 and L2, were also associated with hepatobiliary diseases. Bone isoenzymes B1 and especially B2 were increased in patients with bone diseases (osteomalacia, osteoporosis, and bone metastasis), whereas the B2 activity was predominantly found to be increased in patients with Paget's disease. In three patients we observed an increase of both the bone and liver AP isoenzymes, which might reflect the combination of liver and bone diseases.

We conclude that weak anion-exchange HPLC provides an efficient and sensitive method for the determination of AP isozyme activities, which holds promise as a diagnostic and investigative tool in clinical medicine.

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References

8. Vacik DN, Toren EC. Separation and measurement of isoenzymes.

Table 2. Activity Concentrations and Respective Reference Intervals of AP Isoenzymes in Sera from Healthy Adults

<table>
<thead>
<tr>
<th>Isoenzyme</th>
<th>Mean</th>
<th>SD</th>
<th>Reference Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestinal</td>
<td>4.5</td>
<td>3.9</td>
<td>0–18.9</td>
</tr>
<tr>
<td>Bone B1</td>
<td>3.7</td>
<td>4.3</td>
<td>0–14.2</td>
</tr>
<tr>
<td>Bone B2</td>
<td>5.8</td>
<td>8.6</td>
<td>0–23.1</td>
</tr>
<tr>
<td>Liver L1</td>
<td>33.0</td>
<td>10.6</td>
<td>18.9–62.8</td>
</tr>
<tr>
<td>Liver L2</td>
<td>12.0</td>
<td>4.8</td>
<td>5.0–21.1</td>
</tr>
</tbody>
</table>

*Mean ±2 SD calculated after log-normalization of the data. n = 14.


