Two New Methods for Separating and Quantifying Bone and Liver Alkaline Phosphatase Isoenzymes in Plasma

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We describe two new methods for the separation and quantification of the bone and liver isoenzymes of alkaline phosphatase (EC 3.1.3.1) in plasma. In the first, we use wheat-germ lectin to precipitate the bone isoenzyme. About 80% of this, but minimal liver isoenzyme, is precipitated. The activity of the bone isoenzyme is calculated from measuring the alkaline phosphatase activity in the precipitate, that of liver alkaline phosphatase by subtracting the activity of the bone isoenzyme from total alkaline phosphatase activity. The liver fraction will also contain biliary, intestinal, and placental alkaline phosphatase if these are present in the original plasma, but correction for such activity is readily made. In the second method, samples are separated on cellulose acetate membranes that, before electrophoresis, have been soaked in buffer containing wheat-germ lectin. The bone isoenzyme is retarded and clearly separated from the liver fraction, allowing these isoenzymes to be quantified by densitometry. Both methods are rapid, reproducible, and suitable for use in the diagnostic laboratory.

Additional Keyphrases: wheat-germ lectin · electrophoresis on cellulose acetate · affinity electrophoresis · enzyme activity · reference interval

Lectins are highly specific, carbohydrate-binding proteins of plant origin. Many attempts have been made to utilize them for isoenzyme differentiation, but generally have not resulted in diagnostically useful procedures. At the suggestion of Professor Joachim Kohn we re-examined the effect of lectins on various isoenzyme systems. During our studies we observed a marked differential effect of lectin from wheat {Triticum vulgaris} germ on the plasma isoenzymes of alkaline phosphatase (orthophosphoric-monoester phosphohydrolase [alkaline optimum] EC 3.1.3.1; ALP), with the bone form being preferentially bound. We describe here the use of this lectin in a precipitation procedure and an affinity electrophoresis method for the quantification of the bone and liver ALP fractions of human plasma.

Materials and Methods

Clinical Specimens

We collected blood into tubes containing lithium heparin (Sterilin Ltd., Hampshire, U.K.; 15 int. units of heparin per milliliter of blood), and obtained the plasma by centrifugation. To evaluate the precipitation procedure, we examined plasma samples from 67 healthy student volunteers (40 men, 27 women) ages 20 to 36 years; 39 patients with clinically suspected liver disease, who had increased activities of plasma ALP and aspartate aminotransferase; 20 children hospitalized for minor infections; and 20 women in the last trimester of pregnancy. For the affinity electrophoresis method, we examined plasma samples from 66 of the healthy volunteers and from 44 of the patients with bone or liver disease. Plasma was stored in aliquots at \(-18^\circ\)C and examined within one month of collection. Samples were thawed by standing overnight at 4 \(^{\circ}\)C before assay.

Measurement of Total ALP

We determined total ALP activity at 30 \(^{\circ}\)C using 4-nitrophenyl phosphate substrate and diethanolamine buffer according to the Association of Clinical Biochemists Proposed Method (1) adapted for a discrete analyzer, the ABA 100 Bichromatic Analyzer (Abbott Laboratories, Dallas, TX). In our laboratory the upper normal reference limit for adults is 205 U/L.

Measurement of ALP Isoenzymes

Conventional electrophoresis: For qualitative assessment of the bone and liver isoenzyme composition of plasma, we applied samples to cellulose acetate membranes (Sepaphore III; Gelman Instrument Corp., Ann Arbor, MI) and used the Beckman Microzone electrophoresis apparatus (Beckman RIIC Ltd., High Wycombe, Bucks., U.K.) with Tris/barbital/sodium barbital buffer (Gelman "High Resolution" buffer), pH 8.8, \(I 0.05\), in the buffer compartments.

We electrophoresed 0.25- to 1-\(\mu\)L samples at 250 V for 60 min at 4 \(^{\circ}\)C, using 1-\(\mu\)L samples for total ALP activity up to 800 U/L, and smaller volumes for samples with higher activities. We demonstrated ALP activity after electrophoresis by incubating the membrane for 60 min at 37 \(^{\circ}\)C with a chromogenic substrate, 5-bromo-4-chloro-3-indolyl phosphate, \(p\)-toluidine salt (Sigma Chemical Co. Ltd., Poole, U.K.) (2) at a final concentration of 1.25 mmol/L in 2-amino-2-methyl-1,3-propanediol buffer (1 mol/L, pH 10.2), and magnesium sulfate, 1 mmol/L, in a 10 g/L agar gel (Agar-Noble; Difco Laboratories, West Surrey, U.K.). The stained membrane was then soaked for 5 min in aqueous acetic acid (50 mL/L), washed with distilled water, and dried between weighted-down filter paper. In samples containing intestinal or biliary isoenzymes of ALP, we quantified these isoenzymes by scanning the stained membrane with a densitometer (Model 720; Corning Medical, Medfield, MA).

Sequential heat-inactivation: We also quantified liver and bone ALP activities by sequential heat-inactivation (3), heating the sample at 57 \(^{\circ}\)C for 15 and 25 min. Loss of ALP activity over this period is assumed to be linear and due entirely to liver ALP, so that bone ALP is calculated as total ALP minus the liver fraction.

Lectin precipitation: We prepared in distilled water a 5 g/L (139 mmol/L) aqueous solution of wheat-germ lectin, available as a lyophilized powder from Sigma Chemical Co. or Boehringer Mannheim, Mannheim, F.R.G. To determine ALP isoenzymes, we mixed 50 \(\mu\)L of the lectin solution with 50 \(\mu\)L of plasma. Samples demonstrated by preliminary electrophoresis to contain biliary ALP were pretreated by...
incubating, for 30 min at 37 °C, 50 μL of sample with 5 μL of Triton X-100 surfactant (BDH Chemicals Ltd., Dagenham, Essex, U.K.), 20 g/L in distilled water. The lectin/plasma mixture was incubated for 30 min at 37 °C and centrifuged (2000 × g) for 15 min. We removed all of the supernate by aspiration with a fine-bore pipette and measured its ALP activity. We resuspended the precipitate in 100 μL of saline (sodium chloride, 154 mmol/L of distilled water) and determined its ALP activity. Alternatively, the precipitate may be solubilized in 100 μL of sodium dodecyl sulfate (BDH Chemicals Ltd.), 35 mmol/L in saline solution. Combining the precipitate and supernate activities, and adjusting for sample dilution, yields the total recovery of isoenzyme activity.

About 80% of the bone ALP in plasma is precipitated, whereas precipitation of liver ALP is minimal (see Results). We therefore calculated the bone ALP content of the plasma sample by multiplying the ALP activity of the resuspended or solubilized precipitate by 1.25 (100/80) and adjusting for the initial sample dilution. Subtracting bone ALP from the total ALP activity yields the ALP activity of liver origin (plus biliary, intestinal, and placental ALP when these are present in the original sample). Biliary and intestinal ALP may be identified by preliminary electrophoresis with the conventional method described above (detection limit 5–10 U/L); if their presence is clearly visible, their activity may be quantified by densitometry (4) and deducted from the total ALP activity before calculating the liver ALP activity. Placental ALP, which appears in plasma during late pregnancy, can be determined as the activity remaining in the original sample after heating at 65 °C for 5 min (5).

Affinity electrophoresis: The electrophoretic procedure described above for separation of ALP isoenzymes was modified by including wheat-germ lectin, 50 mg/L (1.39 μmol/L), in the buffer used to soak the cellulose acetate membrane before electrophoresis. We quantified the bone and liver isoenzymes by reflectance densitometry at 600 nm, and expressed the results as percentages of total ALP activity. The activity of each isoenzyme fraction was calculated by multiplying the percentage by the total ALP activity.

Results

Precipitation Procedure

Optimization studies: Optimal conditions for the precipitation method were established from measurements of ALP activity in the precipitate and supernate after lectin treatment. Results were identical for either serum or plasma from the same blood sample and for either resuspended precipitates or those solubilized in sodium dodecyl sulfate. We investigated varying the lectin concentrations between 1.25 and 10 g/L, in either distilled water or saline; incubation times between 5 and 30 min and temperatures between 20 and 37 °C; and addition of polyethylene glycol 6000 (BDH Chemicals Ltd.) up to a final concentration of 20 g/L. The conditions detailed above yielded maximal differentiation between plasma samples from patients with bone and liver disease, which had been shown by electrophoresis to contain predominantly bone or liver ALP, respectively.

Composition of precipitates and supernates from lectin-treated plasma: Butanol or aqueous extracts of bone and liver ALP were less effectively differentiated by wheat-germ lectin than were patients' plasma samples containing these respective isoenzymes, presumably because of alteration in surface carbohydrate during extraction, or because of matrix effects. Thus we quantified the extent of precipitation of the bone and liver fractions from plasma by the lectin by comparison with the results of the sequential heat-inactiva-

tion method. For the comparison, we used 14 plasma samples shown by electrophoresis and by sequential heat-inactivation to contain mainly bone ALP and seven samples that contained mainly liver ALP.

For plasma samples for which 90 ± 7.9% (mean ± SD) of the total activity was bone ALP (by heat-inactivation method), 73 ± 11% of total ALP was precipitated by lectin, whereas for samples with a mean bone ALP of 25 ± 5.4% (heat inactivation) only 21 ± 6.0% of total ALP was precipitated. Extrapolation of these data indicates that, on the average, 80% of bone ALP would be precipitated from plasmas composed entirely of this isoenzyme, whereas the precipitate from plasma containing entirely liver enzyme would show negligible ALP activity. Activity in the precipitates of mixtures of plasma containing the bone and liver isoenzymes in various proportions demonstrated that a constant proportion of bone ALP was precipitated in each case. We concluded that it was appropriate to multiply the precipitate activity by 1.25 to determine the activity of bone ALP in the original sample.

Analytical recovery of total ALP activity from precipitates and supernates combined was 103% for the group of samples containing bone ALP and 110% in the liver ALP group. This indicates some activation of ALP activity, most markedly in the samples containing the highest proportion of the liver fraction.

We further examined the ALP isoenzyme composition of the precipitates and supernates by electrophoresis. To do so, we eluted ALP from the precipitates by adding 100 μL of N-acetylglucosamine (Sigma Chemical Co.), 45 mmol/L in saline solution. Wheat-germ lectin is known to bind to N-acetylglucosamine residues of glycoproteins (6), and we found that the precipitated bone ALP from plasma was fully unbound and solubilized by this concentration of N-acetylglucosamine.

In plasmas with undetectable bone ALP in the original sample we saw none of this isoenzyme in the supernate or the precipitate after lectin treatment. However, in plasmas thought to contain mainly bone ALP, some liver ALP was generally detectable in the supernate after lectin treatment, together with some activity at the origin on the electrophoretogram, presumably from bound but unprecipitated bone enzyme. Only bone ALP was recoverable from the lectin precipitates at activities averaging 83% of total ALP in the three samples examined.

For patients with liver disease, whose plasmas were shown by electrophoresis to contain solely liver and biliary ALP, the lectin treatment did not affect the staining intensity of their supernate liver ALP. Biliary ALP, however, became undetectable in the supernates and the electrophoretograms showed marked staining at the origin, indicating that the biliary fraction was bound but not precipitated. The precipitates from such plasmas showed small amounts of liver and biliary isoenzyme. In the seven samples examined, this precipitated ALP activity averaged 17% of total ALP.

Pretreatment of these samples by incubation with Triton X-100 eliminated the biliary band in the electrophoretograms of the supernates and enhanced liver ALP activity, which suggests conversion of biliary to liver-type ALP by delipidation (7). Electrophoresis of the precipitates showed only a minimal amount of the liver isoenzyme and no biliary ALP, so that the precipitated ALP activity now averaged only 12% of total ALP activity.

We did not detect intestinal ALP in precipitates from samples containing this fraction; it remained in the supernates. Similarly, placental ALP was not identified in precipitates from pregnancy samples. Because the supernates may contain other than liver ALP and may show activation of
ALP by the lectin, whereas the precipitate contains almost exclusively bone ALP, we decided to measure precipitated ALP activity to calculate the bone ALP activity and calculate liver ALP by subtracting bone ALP from total ALP activity, rather than determine liver ALP from ALP activity in the supernate.

Effect of pretreatment with Triton X-100: Because pretreatment with Triton X-100 prevents precipitation of biliary ALP, we recommend it for all samples showing significant activity of this fraction. This pretreatment had no effect on ALP activity in the precipitate in samples containing no biliary ALP. In 18 such samples, least-squares linear regression of the ALP activity precipitates of pretreated (y) and untreated (x) samples yielded an equation of y = 1.02x - 10.52 U/L, S_err = 18.15, with a correlation coefficient (r) = 0.99. In 19 samples with biliary ALP the regression equation was y = 0.92x - 16.03 U/L, S_err = 19.14 (r = 0.99). Comparison of the difference in precipitated ALP activity between these pretreated and untreated samples (mean ± SD, 25 ± 4 U/L) with the biliary ALP activity of the untreated samples (106 ± 78 U/L) indicates that, without Triton X-100 pretreatment, 23 ± 12% of biliary ALP was precipitated.

Precision studies: To determine the within-batch and between-batch precision of this method, we used pooled human plasmas having total ALP activities of 171 and 183 U/L, respectively. The within-batch (n = 24) CV was 3.2% at a mean bone ALP activity of 67 U/L. The between-batch (n = 20) CV was 6.9% at a mean bone ALP activity of 76 U/L.

Comparison studies: Plasma bone ALP activity as determined by precipitation with wheat-germ lectin (γ) was compared with that measured by sequential heat-inactivation (α) for 133 subjects whose bone ALP ranged from about 30 to 3000 U/L (24 patients with bone disease, 50 with liver disease, and 59 healthy volunteers). The correlation was good (r = 0.98, y = 0.97x - 9.96 U/L, S_err = 86.24). A comparison of liver ALP activities by the two methods in the same samples, with liver ALP activities ranging from about 20 to 1000 U/L, gave corresponding values of r = 0.93, y = 1.02x + 14.34, and S_err = 85.86.

Because both the sequential heat-inactivation and the lectin precipitation include both intestinal and biliary ALP in the liver fraction, we applied no correction to the precipitation method to compensate for their presence. Intestinal ALP was detectable by electrophoresis in only six of 74 (8.1%) samples from patients with bone or liver disease and in only 11 of the 67 healthy subjects (16.4%). In the former group the activity of intestinal ALP did not exceed 12% (mean 8.3%) of total ALP or 43 U/L (mean 28 U/L). In the latter group, intestinal ALP activity did not exceed 37 U/L (mean 19 U/L). Biliary ALP was present in 41 patients' samples (mean ± SD 164 ± 127 U/L, representing 28 ± 13% of total ALP activity).

Linearity: The correlation of bone and liver ALP activities by lectin precipitation and by heat-inactivation was linear for total ALP activity up to at least 15-fold the adult upper reference limit, and up to 3000 U/L for bone ALP or 1000 U/L for liver ALP. Diluting the high-activity samples in water, saline, or heated plasma impaired the precipitation of bone phosphatase and is not recommended. For samples containing total ALP activity greater than 3000 U/L, the concentration of lectin in the precipitating solution should be proportionately increased.

Reference values: For the 40 healthy men, total ALP averaged 108 (SD 22) U/L, bone ALP 81 (SD 22) U/L, and liver ALP 27 (SD 9.4) U/L. For the 27 women the corresponding values were 89 (SD 19), 57 (SD 11), and 33 (SD 8.2) U/L. The distribution of enzyme values was gaussian; adjusted for temperature of determination, these values were similar to values reported for this age group by sequential heat-inactivation (δ).

Affinity Electrophoresis

In plasma samples containing both bone and liver ALP, the bone isoenzyme had retarded mobility and was clearly separated from the liver enzyme by the new procedure, whereas with the unmodified conventional method the two fractions were incompletely resolved (Figure 1). Biliary ALP, when present, was bound by the lectin and either remained at or near the origin, in contrast to its α1-globulin mobility, anodal to the liver and bone fractions, by conventional electrophoresis. The mobility of intestinal ALP (normally cathodal to bone ALP) was unaffected. Similar patterns were obtained with either plasma or serum from the same blood sample, although the resolution was best when we used fresh serum. Repeated freezing and thawing impaired the quality of separation.

Optimization studies: Using various concentrations of wheat-germ lectin (between 0.025 and 0.1 g/L) in buffer, we determined that a 50 mg/L concentration best resolved the bone and liver isoenzymes.

Comparison studies: Resolution between the bone and liver fractions by the lectin procedure was such that we could quantify them by densitometry, which we used to compare ALP isoenzyme activity by the new electrophoretic method (γ) with that determined by wheat germ lectin precipitation and by sequential heat-inactivation. Comparison of bone ALP by electrophoresis with that obtained by lectin precipitation yielded a least-squares linear regression equation of y = 1.03x - 19.31 U/L (n = 108, r = 0.99, S_err = 72.03). Comparison of the electrophoretic results for bone ALP activity with those by sequential heat-inactivation yielded y = 1.01x - 24.56 (n = 102, r = 0.99, S_err = 74.56). One sample had negligible bone ALP by sequential heat-inactivation but clearly demonstrable bone isoenzyme by affinity electrophoresis and by lectin precipitation.

The corresponding correlations for determinations of liver ALP were, for lectin precipitation, y = 0.93x + 17.34 U/L (n = 108, r = 0.92, S_err = 70.15) and, for sequential heat-inactivation, y = 0.10x + 16.92 U/L (n = 102, r = 0.90, S_err = 80.01).

For four samples the precipitation technique indicated no liver ALP, and conventional electrophoresis gave no definite indication of liver ALP because of a marked increase in bone ALP. By affinity electrophoresis, however, liver ALP was clearly detectable, albeit at low activity. For patients' sam-

Fig. 1. Separation of plasma samples containing bone (B) and liver (L) ALP isoenzymes by affinity electrophoresis with wheat-germ lectin and by conventional electrophoresis.

α1 = biliary, I = intestinal ALP
amples containing bone ALP, the degree of retardation of that isoenzyme on affinity electrophoresis varied slightly from sample to sample, but in no case overlapped with the liver fraction.

Linearity: The correlation of bone and liver ALP activities by lectin affinity electrophoresis and by lectin precipitation was linear over the same range as that stated above for the lectin precipitation procedure.

Precision: We determined the within-batch and between-batch precision of the method with a plasma sample having total ALP activity of 220 U/L. The within-batch (n = 16) CV was 1.2% at a mean liver ALP activity of 161 U/L and 3.2% at a mean bone ALP activity of 59 U/L. The corresponding between-batch (n = 24) CVs were 2.2% and 5.1%, respectively.

Reference values: In the 40 healthy young men, total ALP activity averaged 108 (SD 22) U/L, bone ALP 63 (SD 18) U/L, and liver ALP 43 (SD 15) U/L. In the 26 women, corresponding values were 91 (SD 17), 47 (SD 11), and 42 (SD 12) U/L. The distribution of enzyme values was gaussian, and these values are similar to those previously obtained by the lectin-precipitation procedure.

Discussion

Alkaline phosphatase is present in tissue-specific ("isoenzyme") forms in liver, bone, intestine, and placenta. In health, plasma ALP originates principally from bone and liver, with a minor contribution (averaging 10% of total activity) from the intestine in approximately one-quarter of all subjects (9). In the latter half of pregnancy, ALP of placental origin may enter plasma.

Measurement of plasma ALP is of established diagnostic value in bone and liver disease. These disorders increase plasma total ALP as a result of increased entry into the plasma of the bone or liver ALP isoenzymes. In addition, in liver disease, biliary ALP (synonyms: high molecular mass ALP; alpha1 ALP; "fast" ALP) derived from cells lining the biliary tract or from the aggregation in bile or plasma of liver ALP with lipid or protein may appear in plasma (7, 10), and plasma intestinal ALP activity may increase as a result of decreased hepatic uptake of this fraction (11).

Approximately half of the clinical requests for ALP isoenzyme determinations are for the differentiation of a bone or liver source for an above-normal activity of total ALP (9). For this purpose, qualitative identification of the isoenzyme composition of plasma is adequate. However, particularly when total ALP activity is within the normal range, quantitative rather than qualitative measurement of bone and liver phosphatase increases the sensitivity of isoenzyme analysis, by permitting detection of minor changes in the activities of these isoenzymes. Quantitative measurements are also essential for the serial monitoring of the activity of these fractions, which is especially required in the follow-up of patients with malignant disease, to identify bone or liver metastases and the response of these to therapy.

Although electrophoresis is convenient for qualitative examination of ALP isoenzymes, bone and liver phosphatases are generally insufficiently resolved by electrophoresis to permit their quantification by densitometry, even though this may be suitable for measuring biliary and intestinal ALP activities.

Of the various nonelectrophoretic methods for quantifying bone and liver ALP activity in plasma (12), most involve use of minor differences in the response of bone and liver ALP to heat or to inhibitors. However, these procedures have proved too complex, insensitive, or unreliable for clinical application; only the use of sequential heat-inactivation has gained any degree of acceptance. We find that this procedure is too tedious and impractical for large sample numbers and that it requires scrupulous procedural control for even moderate reproducibility. In contrast, the precipitation and affinity electrophoresis methods with wheat-germ lectin are very reproducible, give excellent resolution of the bone and liver isoenzymes, readily permit their quantification, and can be carried out in any diagnostic biochemistry laboratory. Because wheat-germ lectin is known to bind to N-acetylgalactosamine residues of glycoproteins (6), the differential effects of the lectin presumably reflect differences in the surface carbohydrates of the bone and liver isoenzymes.

With our precipitation method, a preliminary conventional electrophoresis of ALP isoenzymes is desirable, to identify and quantify the biliary isoenzyme, if present, and to permit quantification of any intestinal ALP, which otherwise would be included with the liver fraction. Note that preliminary electrophoresis is also recommended for the comparison heat-inactivation procedure and for most other non-electrophoretic procedures and that the heat-inactivation method also includes biliary and intestinal ALP with the liver fraction. Biliary ALP is itself of hepatobiliary origin; except in liver cirrhosis, activity of intestinal ALP in plasma is generally too low to significantly interfere and may be further reduced in samples from fasting patients. Nevertheless, separate quantification of these fractions may be of diagnostic value.

When biliary ALP is present, preincubation of the sample with Triton X-100 prevents the precipitation of biliary ALP without affecting the differentiation between the bone and liver isoenzymes. Without such pretreatment, about one-quarter of the biliary ALP would be precipitated and measured with the bone fraction. If samples do not undergo preliminary electrophoresis before precipitation with wheat-germ lectin, all samples should be pretreated with Triton X-100.

As an alternative to the precipitation procedure, modification of conventional electrophoresis by including wheat-germ lectin in the buffer for soaking the cellulose acetate membrane improves the resolution of the bone and liver ALP fractions. In the absence of such modification the two fractions overlap and cannot be quantitated. The mobility of intestinal ALP is unaltered, but biliary ALP, which is of alpha1 mobility by conventional electrophoresis, is markedly retarded and remains at or near the origin. Occasionally one sees faint, diffuse staining between the origin and bone fraction. Consequently, where quantification of biliary or intestinal phosphatase is required, we recommend parallel separation of samples by modified and unmodified electrophoresis.

In conclusion, after treatment with wheat-germ lectin, measurement of ALP activity in the precipitate can be used to quantify bone phosphatase and, by the difference from total ALP, the amount of liver isoenzyme in plasma. The precipitation procedure is preferably preceded by isoenzyme electrophoresis, which can correct the activity of the liver fraction for biliary or intestinal isoenzyme therein. In pregnant subjects, heat treatment of the plasma can correct for placental phosphatase, which otherwise would be included in the liver fraction. Although the precipitation method is the more convenient if quantification of bone ALP alone is required, the affinity electrophoresis procedure improves the electrophoretic resolution of bone and liver ALP such that each can be quantified by densitometry, thus permitting quantification of bone, liver, biliary, and intestinal ALP fractions by electrophoresis alone.
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References


