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Evaluation of serum bone alkaline phosphatase activity in patients with liver disease: Comparison between electrophoresis and chemiluminescent enzyme immunoassay

Fangjie Zhan [a], Yoshihisa Watanabe [a], Aya Shimoda [b], Etsuko Hamada [b], Yoshimasa Kobayashi [c], Masato Maekawa [a,b,*]

[a] Department of Laboratory Medicine, Hamamatsu University School of Medicine, 1-20-1, Handayama, Higashi-ku, Hamamatsu 431-3192, Japan
[b] Department of Clinical Laboratory, Hamamatsu University Hospital, 1-20-1, Handayama, Higashi-ku, Hamamatsu 431-3192, Japan
[c] Second Division, Department of Internal Medicine, Hamamatsu University School of Medicine, 1-20-1, Handayama, Higashi-ku, Hamamatsu 431-3192, Japan

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ABSTRACT

Background: Serum bone alkaline phosphatase (ALP) is a marker of bone formation and metabolism. However, existing methods for measuring it have their limitations and their accuracy has not been determined.
Methods: We measured serum bone ALP activity in 127 patients with liver disease using 2 methods: electrophoresis and chemiluminescent enzyme immunoassay (CLEIA). The results of these 2 methods were compared and analyzed according to gender, age and several serum biochemical markers.
Results: When ALP3 (%; bone-type isozyme activity as a percentage of total ALP activity) values were high, the 2 methods showed good correlation. However, with a decrease in ALP3 (%) levels, the correlation coefficient (R) also decreased. Starting with ALP3 (%) < 23, R values markedly decreased to <0.50 ($p > 0.05$). Five outliers displayed low ALP3 (%) activity levels. Furthermore, in regard to genders, there were significant differences in total cholesterol (TC), γ-glutamyltransferase (γ-GTP), ALP and ALP3 (%) levels ($p < 0.05$).
Conclusions: When serum ALP3 (%) levels were high in patients with liver disease, the accuracy of electrophoresis was comparable to that of CLEIA. However, the accuracy of electrophoresis needs to be evaluated with further when patient samples under certain conditions.

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1. Introduction

Alkaline phosphatase (ALP) is a membrane-bound metalloenzyme that is found in nearly all species [1,2]. Human ALP consists of a group of isozymes, placental, intestinal and three major tissue-nonspecific isoforms [3], which are encoded by 4 main gene loci: tissue-nonspecific, intestinal, placental, and germ-cell ALP [4–6]. Tissue-nonspecific bone- and liver-type ALP are formed through posttranslational modifications and differences in carbohydrate composition, leading to different clinical phenotypes [4]. ALP isozymes have been used as biomarkers for liver and bone disease in the clinic [7–10]. Hence, the measurement of ALP isozymes activity in serum has a high clinical value in the differential diagnosis and analysis of some diseases.

Bone ALP is an extracellular enzyme that is found anchored to the osteoblast membrane and which reflects whole skeletal remodeling [11]; it is one of the most commonly used biochemical markers for osteoblastic bone formation analyzed in the routine clinical chemistry laboratory [12,13]. Metastatic bone tumors, diabetes, hyperthyroidism, fracture recovery period and other conditions lead to the hyperfunction of bone metabolism or formation, which, in turn, increases serum bone ALP levels. In addition, serum bone ALP varies with age and gender. Humans in infancy and at puberty show 2 physiological blood bone ALP concentration peaks, with troughs in mid-childhood and at the end of adolescence [14,15]. Furthermore, for postmenopausal women with high bone turnover, bone ALP concentrations were found to be significantly higher than for those in premenopausal [16–18].

Presently, the predominant methods for separating and measuring bone ALP are electrophoresis and chemiluminescent enzyme immunoassay (CLEIA). The separation of proteins by gel electrophoresis occurs according to their differences in net charge, isoelectric point and molecular weights, resulting in different migration patterns. Migrating in order from the anode to cathode, ALPs were named ALP1 (a high molecular mass, liver-type ALP), ALP2 (liver-type ALP), ALP3 (bone-type ALP), ALP4 (placental-type ALP), ALP5 (intestinal-type ALP) and ALP6

Abbreviations: ALP, alkaline phosphatase; BAP, bone-type alkaline phosphatase; CLEIA, chemiluminescent enzyme immunoassay; TC, total cholesterol; CH, cholesterol; TG, triglyceride; γ-GTP, γ-glutamyltransferase; LAP, leucine aminopeptidase.

* Corresponding author at: Department of Laboratory Medicine, Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan.
E-mail address: mmaekawa@hama-med.ac.jp (M. Maekawa).

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(immunoglobulin binding-type ALP). However, the migration of bone ALP by electrophoresis is also influenced by the presence of other types of ALP isozymes.

The CLEIA technique involves samples binding to mouse monoclonal anti-bone ALP antibodies that, in turn, bind magnetic particles coated with goat anti-mouse polyclonal antibodies. The chemiluminescent substrate (lumigen PPD) emits light according to the activity of bone ALP, with the amount of light emitted reflecting the bone ALP concentration of the sample [19]. CLEIA is widely used in the clinic due to its high sensitivity and specificity, and is simple and rapid [20]. However, the CLEIA method also has disadvantages such as cross-immunological reactivity between bone- and liver-type ALPs, as well as interference by liver-type ALP. CLEIA also does not yield information on other isozymes and isoforms of ALP present within a sample. In fact, the 2 methods have their own distinct limitations when detecting bone ALP.

2. Materials and methods

2.1. Subjects

The subjects of this study were 127 patients (25–83 years; males 54, females 73) with liver disease who were treated at Hamamatsu University Hospital. Of these, 52 patients had primary biliary cirrhosis, 8 were affected by cholestatic cirrhosis, 13 had cholestatic hepatic disorder, and the rest were diagnosed with other liver diseases. Both agarose gel electrophoresis and CLEIA were used to detect bone ALP in serum samples. Other patient biochemical analyses were also undertaken, such as tests for total cholesterol (TC), triglyceride (TG), γ-glutamyltransferase (γ-GTP) and leucine aminopeptidase (LAP), as well as others. The ethics committee of Hamamatsu University School of Medicine approved this study.

2.2. Electrophoretic separation of ALP isozymes

For the analysis of ALP isozymes, electrophoresis using agarose gels was undertaken according to the manufacturer's operating instructions. Quick Gel ALP and Quick ALP reagent (Quick ALP; Helena Labs) have been commercially developed for the electrophoretic analysis of ALP [21]. In order to separate liver- and bone-type ALP completely, samples were pretreated with neuraminidase (ALP separator; Helena) [6]. In addition, samples were pretreated with protease (Quick ALP; Helena) to separate bone- and intestinal-type ALP. Following electrophoresis, a 3-indoxyl phosphate disodium salt was used as a substrate and nitrotetrazolium blue was used as a chromogenic agent. Bands were scanned by densitometry at a wavelength of 570 nm [21]. ALP isozyme activity was expressed in U/l and as a percentage of total ALP activity. Samples from 3 healthy individuals were measured using three lots of reagents, indicating the number of assays for reproducibility analysis totaled nine times. The coefficients of variation (CVs) for ALP3 activities were 1.22–3.00%. However, these were not the CVs for ALP2 and ALP3 percentages, which are quite different samples.

Serum samples and protease were mixed using a ratio of 5:1 at room temperature for 30 min; both serum and protease-treated samples, respectively, were mixed with neuraminidase at a ratio of 7:1, and left at room temperature for 12 min. Control, serum, separator-treated, protease-treated and separator + protease-treated samples were loaded in order on the gel plate. Electrophoresis was performed at 250 V for 20 min. The gel was coated in sample reagent and stained for 1 min before incubation at 37 °C for 35 min. The gel was soaked in 70% methanol for 10 min and washed with water twice for 10 min each wash. Bands were then scanned by densitometry at a wavelength of 570 nm.

2.3. CLEIA

A 1-step immunoenzymatic assay was used in this study. An automated chemiluminescence enzyme immunoassay device, known as an Access Immunoanalyzer (Beckman Coulter), and a special bone-type ALP (BAP) kit (Access Ostase; Beckman Coulter) were used [19,22]. Beckman BAP Access Ostase operating instructions were followed. Samples and reagents were mixed, and all operations were automatically performed as long as the appropriate operating items were chosen. Each 25 μl sample was mixed with 50 μl of a solid phase reagent (magnetic particles coated with goat anti-mouse polyclonal antibodies) and 15 μl of an antibody reagent (mouse monoclonal anti-BAP antibodies), and then washed after reacting at 37 °C for 14.4 min. Next, a 200 μl substrate solution (lumigen PPD) was added and samples incubated at 37 °C for 5 min. After each reaction was complete, its luminous intensity (λ max = 540 nm) was assayed by immunoanalyzer and the concentration of BAP in the sample was automatically calculated using a standard curve. Assays were repeated 3 times in reproducibility analysis, for which intraday and interday CVs for BAP were 3.82–5.79% and 2.60–5.25% for a 10.39 μg/l concentration, 3.77–5.66% and 3.33–5.80% for 28.07 μg/l.

2.4. Serum biochemical examinations

Basic biochemical examinations of serum samples, using a chemistry analyzer (Labospect 008; Hitachi Ltd), were undertaken. Cholesterol (CH), TG and γ-GTP assays were performed according to the manufacturer's operating instructions. To determine CH and TG fractions in serum, serum lipoproteins were separated by electrophoresis using agarose gels. After electrophoresis, reagents (Choletricombo CH/TG; Helena) within the agarose reacted with CH and TG in lipoprotein fractions, and the formazan consequently produced was scanned by densitometry at a wavelength of 570 nm. For the measurement of serum γ-GTP isozymes, a cellulose acetate membrane was used as a support and soaked in Tris-barbital buffer (pH 8.8) before serum γ-GTP isozymes were separated by electrophoresis. After electrophoresis, reagents (Titan γ-GTP; Helena) within the acetate membrane generated azodyes that were scanned by densitometry at a wavelength of 570 nm.

2.5. Statistical analysis

All statistical analyses were performed using SPSS software (v18.0, SPSS Inc.). Data are expressed as mean ± SD. Correlations between ALP3 (bone ALP determined by electrophoresis) and BAP (bone ALP measured by CLEIA) were determined using Pearson's correlation coefficient. Differences in means between methods were evaluated by Student's *t*-test. A $p < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Determination of serum bone-type ALP by 2 methods

We used 2 methods, electrophoresis and CLEIA (Suppl. Fig. 1), to detect bone ALP in the serum samples of 127 patients with liver disease (Suppl. Table 1). A comparison of the 2 methods was made, with CLEIA used as a control method and electrophoresis evaluated in order to assess its accuracy in the determination of bone ALP. Fig. 1 shows how bone ALP samples analyzed by electrophoresis (ALP3) compared with those analyzed by CLEIA (BAP). The linear equation was calculated as $y = 8.63x - 44.9$, with a correlation coefficient (R) of 0.886; The 2 methods showed a statistically significant correlation ($p < 0.001$). Furthermore, results for some samples deviated from those of most samples (black triangles), with all outliers below the line.

3.2. Further stratified analysis of all samples

We grouped all samples according to their percentage of ALP3 in 20% increments: >80% (n = 8), 60–80% (n = 18), 40–60% (n = 42), 20–40% (n = 41) and <20% (n = 18), in a total of five groups as shown in Fig. 2.

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