Original Article

Quantification and comparison of bone-specific alkaline phosphatase with two methods in normal and paget's specimens

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Abstract

Background: Bone-specific alkaline phosphatase (BAP) is synthesized by the osteoblasts and is presumed to be involved in the calcification of bone matrix, though its precise role in the formation process is unknown. The aim of the present study was to measure the BAP activity in Paget's and normal specimens by two different techniques.

Methods: Total ALP (TAP) as well as BAP activity-measuring tests were repeatedly undertaken at different times during the day and different days on the serum samples (inter and intra assay). Precision and repeatability of the phenylalanine inhibition (PHI) and heat inactivation (HI) techniques were approved during ten times repetition of all the tests on two normal samples besides one sample from Paget's disease of bone. The measurement of TAP and BAP activities was also carried out on 50 serum samples from normal adults using the standard IFCC-AACC and the established methods, respectively.

Results: Coefficients of Variation (CV) for intra-assay of BAP were 2.33% and 3.16% by HI and PHI methods, respectively. Also, the inter-assay CV of BAP was 2.87% and 3.49% for mentioned methods in Paget's sample, respectively. In addition, the correlation of HI and PHI methods was found to be r= +0.873 for bone-specific isoenzyme.

Conclusion: Regarding the appropriate precision, repeatability and correlation of HI and PHI techniques, as well as their cost effectiveness can be of use in the quantification of bone alkaline phosphatase isoenzyme activity, especially when bone is involved.

Keywords: Bone-specific isoenzyme, Bone marker, Phenylalanine inhibition, Heat inactivation, Paget's disease

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Bone-specific alkaline phosphatase (BAP) is synthesized by the osteoblasts and is presumed to be involved in the calcification of bone matrix, though its precise role in the formation process is unknown. In the serum of most healthy individuals, bone and liver isoenzymes of the tissue non-specific AP gene predominate in approximately equal proportions. ALP isoenzymes mainly exist in bones, liver, intestine, placenta, mammary glands and kidneys. ALP is a commonly encountered laboratory value that is included in the panel of bone and liver function tests. Although, the elevated concentrations generally are attributed to either bone or liver sources (1-5). BAP is considered to be a highly specific marker of the bone-forming activity of osteoblasts. Among the different types of bone diseases, the highest amount of serum ALP activity is observed in Paget's disease which is caused by osteoblasts action following bone destruction by the uncontrolled activity of osteoclasts. Under this condition, ALP activity is almost 10 to 25 times higher than the normal limit. The moderate increase in ALP activity is observed in osteomalacia, which is slowly decreased towards normal ranges in response to vitamin D therapy. The ALP activity rate is generally normal in osteoporosis; while in rickets disease, a 2 to 4 times increase is seen in the enzyme activity, which gradually moves toward the normal range following vitamin D therapy.

Very high levels of ALP enzyme activity is also found in patients with bone metastatic carcinoma and osteogenic sarcoma. Temporary increase in the enzyme activity may be observed during the healing of bone fractures (3-7). A strong association between BAP levels and rapid bone loss was reported from the Hawaii Osteoporosis Study cohort. The association between BAP and bone loss is equivalent between BMD and fracture and suggests the marker can play a valuable role in risk assessment (7).

Physiological bone growth contributes to elevated serum alkaline phosphatase activity and, hence, the level of serum ALP activity is 1.5 to 2.5 times higher in growing children than in normal adults. On the contrary, decreased alkaline phosphatase activity has been observed in cases such as cessation of bone growth, achondroplasia, and cretinism; it has also been reported in hereditary hypothyroidism, hyperthyroidism, scurvy, severe anemia, kwashiorkor, presence of radioactive materials in bone, vitamin B_{12} deficiency (pernicious anemia), and multi-nutritional deficiency of zinc or magnesium (8-11). The measurement of serum alkaline phosphatase activity is of high significance for the evaluation of two groups of conditions including hepatobiliary and bone diseases associated with increased osteoblastic activity. In cases of simultaneous presence of bone and liver disorders, measuring the activity of different isoenzymes has high clinical value, indicating the rate and the extent of the relative tissue damage.

Despite the importance of measuring the different isoenzyme activities in different diseases, total ALP test is often requested, which is not specified to identify the type and the extent of tissue damage. ALP activity-measuring methods have a long history, and several techniques in this field are more or less worthy. Such technical diversity is indicative of the problems existing in the comparison of alkaline phosphatase test results from the different laboratories or reports in literatures. The technical development in this period has moved towards increased test speed, sensitivity and specificity, and identification and facility of substrates hydrolysis; low cost and simplicity of the method are worth mentioning as well. Several methods have been reported for measuring the ALP isoenzymes (1, 12-21). The aim of the present study was to establish and compare HI and PHI methods in the measurement of bone ALP isoenzyme activity as specific and low-cost tests so they can be applicable in all hospitals and clinical laboratories.

Methods

From July 2010 to April 2011 venous blood sample was taken from 50 normal adults in the fasting state. Then, total ALP (TAP) and bone-specific ALP (BAP) isoenzyme methods repeatedly undertook at different times during the day and different days on serum samples, and affecting conditions on the tests results were evaluated. After the establishment of heat inactivation (HI) and phenylalanine inhibition (PHI) techniques, precision and repeatability of the results obtained were investigated using the two normal samples besides one serum sample from a patient with Paget's disease of bone. All the tests were repeated ten times on each of the study samples, and the results were analyzed by SPSS statistical software and the repeatability test, and appropriate accuracy and repeatability of the mentioned tests were statistically confirmed. Afterwards, 50 serum samples of normal adults were evaluated in terms of TAP activity, using the standard IFCC (International Federation of Clinical Chemistry) and AACC (American Association of Clinical Chemistry) methods, and bone-specific ALP activity, using HI and PHI techniques (22).

1) Quantification of bone-specific ALP isoenzyme activity using the heat inactivation (HI) technique: HI method is based on one of the most important features of ALP isoenzymes, i.e. the difference in isoenzymes deactivation rate at different temperatures. The implementation of HI method requires a very precise control of experimental conditions such as temperature and duration of incubation. The part of each sample was placed in a water bath for 5 minutes at 65° C and was immediately cooled in the ice box after this period. This stage of thermal incubation leads to inactivation of bone, liver and intestinal isoenzymes to 100 percent; however, more heat-resistant isoenzymes, such as placental and neoplastic isoenzymes, if present in the samples, remain active under this condition. The other part of each sample was placed in a water bath for 16 minutes at 55°C and was immediately cooled in the ice box following this period. The inactivation rate under this circumstance is 95%, 60% and 55%, respectively for bone, liver and intestinal isoenzymes according to the information in the reference sources (1, 3).

2) Quantification of bone-specific ALP activity using the phenylalanine inhibition (PHI) technique: PHI method is based on the difference in deactivation rate of ALP isoenzymes in the vicinity of a certain concentration of phenylalanine solution. In this approach, part of the serum

sample was incubated for 20 min at 37°C with 11 mM phenylalanine solution. After the incubation, residual ALP enzyme activity in the relative sample along with other parts of sample, undergoing thermal and/or non-thermal stage, was calculated under the same experimental condition using the standard method. Bone and liver ALP isoenzymes are respectively inhibited to 21% and 20% during incubation with 11 mM phenylalanine solution for 20 min, while the other isoenzymes are strongly inhibited in these conditions (1, 3). The amount of bone-specific ALP activity was calculated with respect to total ALP activity.

3) Quantification of TAP enzyme activity: TAP activity was measured using the standard IFCC-AACC method. In this approach, ALP enzyme in the serum sample hydrolyzes the colorless substrate, 4-nitrophenyl phosphate (4-NPP), under PH=10.3 and at 37° C temperature, and the phosphate group released is taken by the buffer 2-Amino-2-methyl-1-propanol (AMP). The product, 4-nitrophenol, is converted to the 4-nitrophenoxide in the reaction pH, which is in equilibrium with its yellow ketonic form. After exactly 15 minutes since the substrate addition, the pH of the reaction medium was brought to about 12 by adding the alkaline solution of sodium hydroxide, halting the enzyme activity and enzymatic reaction. The product's color intensity was read by the spectrophotometer at 405 nm, and the total ALP activity was calculated.

Coefficients of variation of inter and intra assays: Precision implies repeatability, which means analyzing repeatedly to determine variation. For intra-assay variation, one Paget's sample and one normal sample were run 10 times in each day. To verify precision, the Paget's and normal samples were processed 2 times per run for 10 days in one month, generating 20 replicates for each. This is called inter-assay variation.

Precision can be specified as: (i) repeatability (within run), (ii) intermediate precision (long term) (23).

Imprecision is quantified by calculating the mean, standard deviation (SD), and coefficients of variation (CV) of the data collected from an analytical run:

CV = (SD × 100) / Mean

Statistical Analysis

Bone-specific ALP (BAP) and total ALP (TAP) activities in serum samples were expressed as mean±SD. Coefficients of variation (CV) for inter and intra assays were calculated. The results of HI and PHI techniques were subjected to linear regression and Pearson correlation analysis.

Results

Intra-assay variations for bone-specific ALP isoenzyme in the Paget's and normal samples by HI and PHI methods was shown in table 1. Also, table 2 showed that the verification of inter-assay variations for bone-specific ALP isoenzyme in the Paget's and normal samples. Moreover, the correlation of phenylalanine inhibition technique and heat inactivation method was found to be r= +0.873 for bone-specific ALP isoenzyme.

In the present study, 50 normal adults participated with age ranging from 19 to 40 years old, 19 were males and 31 were females. In terms of blood groups, the subjects' frequency was respectively 64% (O^+), 12% (B^+), 12% (AB^+), 10% (A^+), and 2% (A^-).

The mean±SD of TAP activity in 50 normal samples was 91.2±24.7 using the IFCC-AACC standard method. Also, the mean±standard deviation was 46.60±17.54 and 44.26±19.33 for bone-specific ALP isoenzymes, using HI and PHI techniques respectively. Moreover, the comparison of TAP and bone-specific ALP isoenzyme activity using 10 times repetition with IFCC, HI and PHI techniques in normal and Paget's samples are shown in figures 1 and 2, respectively.

Table 1. Intra-assay* variations for Bone-specific ALP isoenzyme (U/L) in normal and Paget's samples by heat inactivation (HI) and phenylalanine inhibition (PHI) methods

Methods	Paget's Sample		Normal sample	
	Mean±SD	CV(%)	Mean±SD	CV(%)
HI	452.30±8.24	2.33	38.90±1.63	2.85
PHI	460±9.83	3.16	40.72±2.05	3.54

* Each sample was run 10 times in one day.

Table 2. Inter-assay* variations for Bone-specific ALP isoenzyme (U/L) in normal and Paget's samples by heat inactivation and phenylalanine inhibition methods

Methods	Paget's Sample		Normal sample	
	Mean±SD	CV (%)	Mean±SD	CV (%)
HI	457.19±10.75	2.87	40.35±2.47	3.12
PHI	463.66±12.05	3.49	42.05±3.18	4.30

* Samples were processed 2 times per run for 10 days during one month, generating 20 replicates for each.

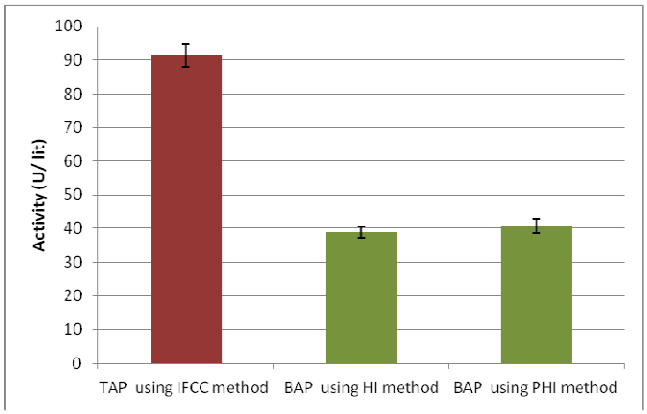

Figure 1. Comparison of TAP and bone-specific ALP isoenzyme activity using 10 times repetition with IFCC, HI and PHI techniques in normal samples

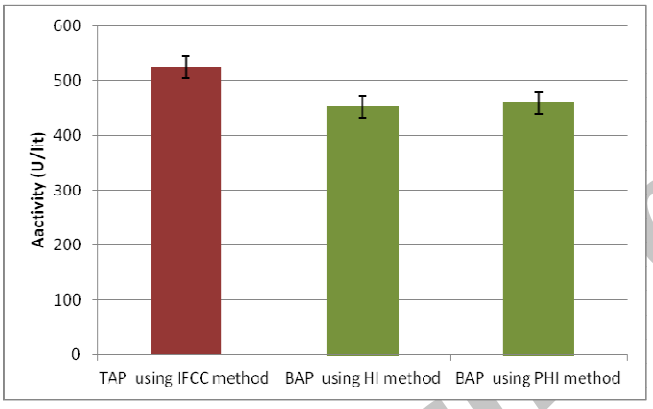

Figure 2. Comparison of TAP and bone-specific ALP isoenzyme activity using 10 times repetition with IFCC, HI and PHI techniques in Paget's samples

Discussion

Several methods have so far been suggested for the quantification of TAP and its isoenzyme activity (1, 12-21). In the present study, TAP has been measured using the standard method proposed by the International Federation of Clinical Chemistry (IFCC) and the American Association of Clinical Chemistry (AACC), possessing more credibility than other existing methods (22).

Various methods have also been reported for the measurement of ALP isoenzyme activity; most of these techniques, such as monoclonal antibody and immunoradioassay (IRMA), require specific equipment and are not cost-effective (16, 17, 21). According to a research conducted, the correlation between the results of IRMA and heat inactivation method has been found to be r = 0.673 (24). In heat inactivation (HI) technique, the precise determination of the reaction temperature and incubation duration are highly important, so as temperature fluctuations and slight changes in the incubation time can cause considerable error in the method outcomes. There is no general consensus among the researchers in selecting a particular temperature and a specified time in heat inactivation technique, although relative deactivation of ALP isoenzymes has been identified for each temporal and thermal condition in the mentioned approach. One of the conditions reported 65°C temperature for 10 min, during which more resistant isoenzymes that retained 90% of their activity such as placental and neoplastic isoenzymes (1, 25).

In this study, two thermal stages were applied with a specific incubation time. Part of each sample was incubated at 65°C for 5 min, under which bone, liver and intestinal isoenzymes lose 100% of their activity, and in cases of remaining ALP activity in the sample serum, the presence of placental or neoplastic isoenzymes can be suspected; however, to ensure the presence of these isoenzymes, the serum sample should be incubated at 65°C for 30 min. The other part of each sample underwent thermal condition at 55°C for 16 min, in which only about 5 percent of bone isoenzyme activity remained, while liver and intestinal isoenzymes maintained 40 and 45 percent of their activity, respectively. However, the possible minor activity of intestinal isoenzymes can be ignored through sampling in fasting state.

In normal serum samples, neoplastic isoenzymes such as Regan do not exist, and placental isoenzymes are only present in women's serum during pregnancy and, therefore, the amount of bone and liver isoenzyme activity can be calculated in this situation compared to total ALP, which is separately measured in each serum sample using the standard method (1, 12, 15, 16). Besides high acceptability and credibility, this measuring method has been confirmed in the reference resource (15, 22).

The study findings demonstrated that phenylalanine inhibition (PHI) technique possesses high precision, repeatability and correlation in the measurement of bone-specific ALP (BAP) isenzyme activity in comparison with HI method. The serum ALP activity has been shown to be significantly associated with age, so as the activity is approximately 5 times higher in infants and about 2.5 times higher in children than adults, which is mainly due to a sharp

increase in the bone isoenzyme activity during growth. The enzyme activity also varies in the same age with respect to gender and is slightly higher in men than women, although it is of minor importance in comparison with age. Furthermore, alkaline phosphatase activity depends on type of blood groups, and part of total ALP activity after meals in subjects with O or B blood groups is related to the intestinal isoenzyme (5, 6, 10, 22).

BAP shows no significant circadian variation in serum as reported by many studies (8). In contrast to total alkaline phosphatase, BAP levels are in general unaffected by diet (26). Since the statistical parameters such as age, gender, blood groups, and experimental conditions were identical in the two established methods in the present study, they had no intervention in the research. In addition, owing to sampling in fasting state, the amount of intestinal isoenzyme activity is insignificant in this condition, and more than 95% of total ALP activity is associated with liver and bone isoenzymes (5, 6, 10, 12, 22). Some researchers have used different concentrations of urea, as an inhibitor, in the measurement of bone and liver isoenzymes activity; some, for example, have applied 2.9 M urea solution (20). In another study by an author, the serum samples were incubated with 3M urea solution at 37° C for 18 min, in which the remaining activity of bone and liver isoenzymes was respectively 16 and 44 percent, the activity of other isoenzymes is not considerable in fasting state and in normal samples (12).

As also reported by reliable sources (15, 22), the most appropriate concentration of phenylalanine in the inhibitory reaction is 11 mM for 20 min. The mentioned concentration of phenylalanine was similarly used in the present study. The results of 10 times repetition of TAP as well as BAP activity-measuring tests in normal serum samples and those obtained from the patient with Paget's disease were mainly within one standard deviation from the mean, indicating a good precision and repeatability of the results achieved. High correlation between the two methods applied, HI and PHI, along with acceptable precision and repeatability and easy application in all laboratories with no need to specified equipment and high-cost techniques such as immunoradioassay and / or monoclonal antibody. The quantification of bone-specific alkaline phosphatase isoenzyme activity is of high clinical value and indicative rate and the extent of the relative disease (7, 8, 10).

Regarding the appropriate precision, repeatability and correlation of HI and PHI techniques, as well as their cost effectiveness, they can be of use in the quantification of bone alkaline phosphatase isoenzymes activity, especially when bone is involved.

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