Effect of incubation period on measurement of alkaline phosphatase enzyme activity at 37°C

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ABSTRACT

Background: Laboratory analysis of alkaline phosphatase (ALP) from blood can detect abnormalities which reflect changes in certain health conditions, including liver disease and bone disease. Despite the importance of measuring different isoenzymes activity in different diseases, total ALP test is often requested, which is not specific to identify the type and the extent of tissue damage. ALP activity measurement at different incubation period may affect the actual measurement as ALP is a heat labile enzyme and specific ALP isoenzymes have different sensitivity to heat inactivation which creates lab to lab variability and significant change if particular isoenzyme is predominant in patient's sera. **Objectives:** The study was performed to measure the loss of activity of ALP at different 37°C incubation periods during ALP measurement in serum. **Materials and Methods:** Quality control materials were analyzed for ALP enzyme activity in 2-amino, 2-methyl, 1-propanol buffer, and 4-nitrophenyl phosphate substrate with different incubation periods. **Result:** There is a gradual decrease in enzyme activity on increase of incubation period. **Conclusion**: The effect of incubation period on ALP activity measurement at 37°C may be clinically relevant if one of the isoenzymes is predominant in the patient sera.

KEY WORDS: Enzyme Measurement; Alkaline Phosphatase; Heat Inactivation; Incubation

INTRODUCTION

Alkaline phosphatase (ALP) isoenzymes mainly exist in bones, liver, intestine, placenta, mammary glands, kidneys, and leukocytes. In normal serum, more than 90% of total ALP activity is related to bone and liver isoenzymes. The relative proportion of the individual enzyme in serum is related to the severity of lesion of the tissue from which it is originated. Thus, one cannot predict the relative proportion of the different forms of enzyme in patient's body. Assessment of ALP activity from blood can detect abnormalities in certain health conditions, including liver disease and, bone disease. Although the importance of measuring different isoenzymes activity in different diseases can reflect particular underlying condition, total ALP test is mostly requested, which is not specific to identify the type and the extent of tissue damage. ALP is a heat labile enzyme and specific ALP isoenzymes have different sensitivity to heat inactivation, so ALP activity measurement at different incubation period will affect the actual measurement as and creates lab to lab variability and significant change if particular isoenzyme is predominant in patient sera.

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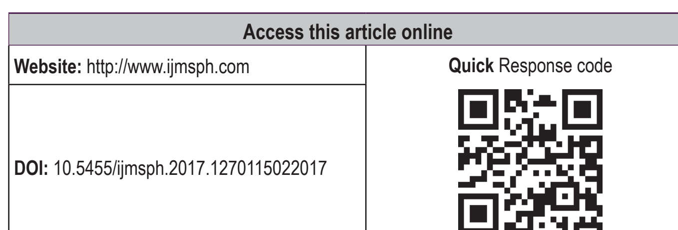

Various methods have been reported for measuring ALP isoenzymes, among which immunoradioactive assay,[1,2] immunoassay,[3] isoelectric focusing,[4] electrophoresis techniques,[5,6] monoclonal antibody,[7] inhibition with chemical compounds such as urea,[8] heat inactivation and precipitation techniques[8,9] can be enumerated.[1]

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Heat inactivation is one of the most important features of ALP isoenzymes. Based on which a method to measure ALP isoenzyme activity is developed, i.e., difference in deactivation rate at different temperature. Implementation of heat inactivation method requires very precise control of experimental conditions such as temperature and incubation period. Incubation is done at 65°C for 5 min. This stage of thermal incubation leads to inactivation of bone, liver, and intestinal isoenzyme to 100%. However, more heat resistant isoenzymes like placental and neoplastic isoenzymes, if present in sample, remain active at this condition.[1] The other way of estimation is to incubate the sample for 16 min at 56°C. The inactivation under these circumstances is 95%, 60%, and 55% respectively for bone, liver, and intestinal isoenzymes.[10]

This study is centered to see the effect of the incubation period on ALP enzyme activity at 37°C.

MATERIALS AND METHODS

The study was performed in Clinical Biochemistry Laboratory, New Civil Hospital, Surat by measuring ALP activity in 2-amino, 2-methyl, 1-propanol (AMP) buffer and 4-nirophenyl phosphate substrate with different incubation periods at 37°C.

Instrumentation and Equipment

Spectrophotometer (Erba XL-640) suitable for measurement of absorbance at wavelength of 415 is used. The specification of the instrument includes sample and reagent handling, temperature control and overall photometric performance according to the International Federation of Clinical Chemistry consideration.

All glassware used in the preparation of reagents and pipetting are calibrated gravimetrically by Shimadzu weighing scale which is calibrated by sartorius lose weights (10 mg, 100 mg, 1 g) which are calibrated externally by the National Accreditation Board for Testing and Calibration Laboratories (NABL) accredited calibration laboratory. pH meter was calibrated using three primary pH standards.

Reagent preparation

All solutions are prepared in calibrated flask with fresh reagent grade analytical grade chemicals.

1. 2A2MP - 900 mmol/L; 86.45 g of 2A2MP added to be dissolved in 800 ml of water
2. 4-Nitrophenyl phosphate - 16 mmol/L; 5.93824 g of 4NPP.6H_2O added to be dissolved in 2A2MP solution
3. Magnesium ion - 1 mmol/L; 203.302 mg of MgCl2.6H_2O added to be dissolved in 2A2MP solution
4. pH was obtained 10.5 (add 10% of HCl if needed) and made up to 1000 ml.

Sample preparation

Lyophilized assayed human sera level 2 (lot = 1029 UN) and level 3 (lot = 767 UE) of RANDOX manufacture were reconstituted with 5 ml deionized water using 5 ml TC volumetric flask calibrated gravimetrically by Shimatzu weighing scale which is calibrated by sartorius lose weights (10 mg, 100 mg, 1 g) which are calibrated externally by NABL accredited calibration laboratory.

Sample Analysis

Following parameters were set to analyze ALP enzyme activity in Erba XL-640 (Table 1).

To measure enzyme activity at different incubation period, five different profiles with different reading cycles and different incubation period were set. Samples were analyzed by all five profiles and absorbance were measured in triplicate. Average of triplicate absorbance was used (Table 2).

Statistical Analysis

Results obtained were exported in spreadsheet, and data were sorted according to different incubation periods and averages of the absorbance of each sample for respective incubation periods were calculated and plotted on graph.

RESULTS

On analyzing samples of human assay control level 2 and human assay control level 3 with different incubation periods, results obtained are shown in Tables 3 and 4, respectively.

As shown in Tables 3 and 4, there is an average 3-4% loss of ALP activity per minute at 37°C.

Figures 1 and 2 show incubation period versus Δabsorbance for Randox assayed human sera Level 2 (lot = 1029 UE) and Randox assayed human sera Level 3 (lot = 676 UE), respectively.

DISCUSSION

From the current study, we have noticed a constant decline in ALP activity on continuing incubation 37°C on both the human assay control sera Level 2 and Level 3. As shown in illustrations 1 and 2, there is decrease in delta absorbance per minute with increasing incubation period for both the QC analyzed. Hence, an average 3-4% of ALP activity is lost per minute if the incubation period at 37°C continues. This indicates inactivation of enzyme at 37°C on increasing incubation period.

Moss and King[11] reported the greater heat stability of liver phosphatase as compared with bone phosphatase. Many reports have appeared of the use of selective-inactivation techniques

Table 1: Test parameter - ALP analysis

Instrument	Erba XL 640	Principle	Diffraction spectrophotometry
Method	AMP optimized to IFCC at 37°C		
Wavelength primary	415	Wavelength secondary	NA
Assay type	Rate-A	Curve type	K-Factor
Reaction direction	Increasing	Incubation period	See Table 2
Sample volume	8 ul	Reagent	200 ul

IFCC: International federation of clinical chemistry, AMP: 2-Amino, 2-methyl, 1-propanol, ALP: Alkaline phosphatase

Table 2: Testing profiles with different incubation period

Profile No.	Incubation cycles	Incubation period (s)	Reading cycles	Reading period (s)
1	1-5	36	5-12	63
2	1-15	126	15-22	63
3	1-24	216	25-32	63
4	1-34	306	35-42	63
5	1-44	396	45-52	63

One cycle=9 s

Table 3: Loss of activity per min for assayed human sera Level 2 (lot=1029 UN)

Incubation period	Delta absorbance	% of activity (36 s incubation=100%)	Loss of activity per min at 37°C
36	0.1021	100	
126	0.0952	93	−4.65
216	0.0894	88	−3.61
306	0.0846	83	−3.13
396	0.0802	79	−2.89
		Average loss of activity per min	−3.57

Table 4: Loss of activity per min for assayed human sera Level 3 (lot=767UE)

Incubation period	Delta absorbance	% of activity (36 s incubation=100%)	Loss of activity per min at 37°C
36	0.1962	100	
126	0.1814	92	−5.04
216	0.1723	88	−3.09
306	0.1570	80	−5.18
396	0.1526	78	−1.49
		Average loss of activity per min	−3.70

including the heat inactivation to provide qualitative or semi-quantitative estimates of the relative proportions of liver and bone phosphatases in serum like studies done by Posen et al.,[12] Fitzgerald et al.,[13] Kerkhoff,[14] and Cadeau and Malkin[15] also reported the selective heat inactivation and loss of particular ALP isoenzyme activity at different temperature and incubation. These reports differ in the choice of heat or urea as the inactivating agent and in other conditions such as temperature. However, in a typical procedure, a residual activity of <20% after 10-mm incubation at 56°C suggests that the specimen originally contained predominantly bone phosphates indicating heat stability of particular isoenzyme as compared to others. Neale et al.[16] reported specific heat stability of placental ALP. Hence, many researches in past already confirmed the heat labile nature of ALP and selective heat stability of different isoenzymes already and that has led to the development of specific heat inactivation techniques for ALP isoenzymes in advanced laboratories. Still specific isoenzyme estimation is advised in very few instances in countries like India. Rej[17] from New York done similar sort of study like ours and investigated effect of incubation as on the ALP measurement with Mg^{2+} on lyophilized control serum. He investigated sera at the following temperatures: 37°C, 21°C (room temperature), 4°C, and −10°C. The results demonstrate that Mg^{2+} can be maintained in the ALP buffer medium for 48 h if it is stored at or below 22°C. Storage at 4°C or −10°C prolongs the useful life of these solutions to longer than 3 weeks; storage at 37°C markedly reduces stability.

The current study is done to evaluate the effect of prolonging the incubation period at particular 37°C on ALP activity. Up till now to our knowledge various studies and research in the past done for different temperature effect like at room temperature (22°C), at 4°C, at (−10°C), at 56°C, at 65°C, and they confirmed loss of ALP activity with rising temperature. Very few studies are available with prolonging incubation at 37°C which can be of useful because such a minor step of following particular incubation time will avoid lab to lab variability in ALP measurement and also has further clinical application. As different ALP isoenzymes have different rate of inactivation on heat exposure, different incubation period used during ALP assay can have different effect on laboratory results, depending on the predominant isoenzyme present in the patient sera. This may affect the patient profile particularly when particular isoenzyme is predominant in patient's sera. The current study is done on lyophilized assayed human control sera Level 2 and Level 3 with normal and high levels of ALP in control sera. Hence, the further research and large data is needed from such studies on patient's sera. Furthermore, the study does not comment on the assessment of particular isoenzymes of ALP. It focuses mainly on the period of incubation at 37°C and its effect on final measurement of total ALP.

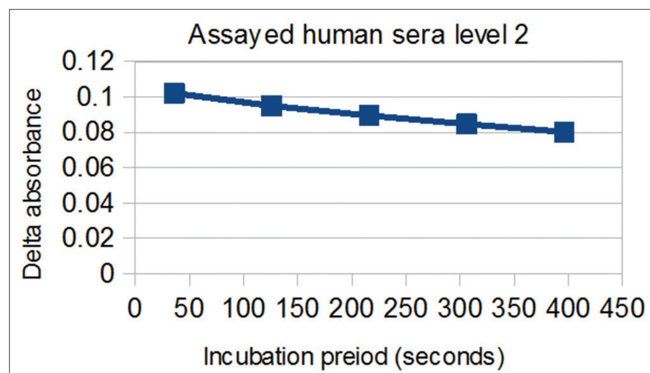

Figure 1: Effect of incubation period on enzymatic activity of alkaline phosphatase on human assay control Level 2

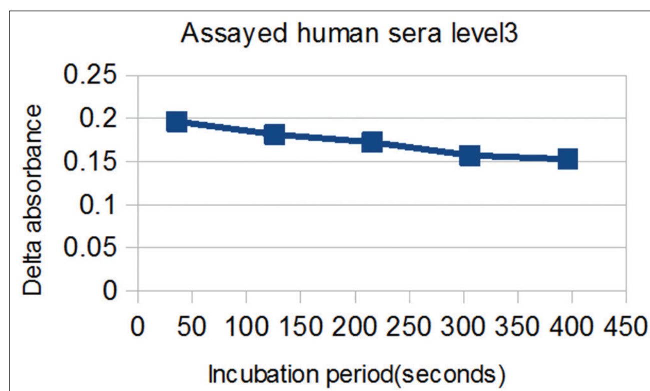

Figure 2: Effect of incubation period on enzymatic activity of alkaline phosphatase on human assay control Level 3

Laboratory must use as little incubation period as possible for avoiding denaturation of enzyme during ALP assay. Moreover, laboratory using manufacture supplied factor must ensure that the incubation period used by the laboratory is validated by the manufacturer. From the current study, we hereby advise to follow the strict incubation period, and manufacturers validated guidelines. The clinician should also keep in mind the fact of ALP inactivation at 37°C when laboratory results of the same patient in different laboratories are compared.

CONCLUSION

ALP activity gradually declines with continued incubation and destroyed at a rate of 3-4%/min at 37°C. Such inactivation may differ across various isoenzymes due to specific heat stability of different isoenzymes. The heat sensitivity of ALP at 37°C has analytical and clinical application, which should be kept in mind and care should be taken by the laboratories for the patient care and quality purpose.

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