

Original Article

Interfering effect of bilirubin on the determination of alkaline phosphatase

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Abstract: Objective: This study was designed to evaluate whether the high concentration of bilirubin is able to interfere the determination of alkaline phosphatase (ALP). Methods: Clinical tests evaluating the interfering substance of bilirubin of various concentrations on the determination of ALP were conducted based upon the Clinical and Laboratory Standards Institute (CLSI) document EP7-A2, the most recent guideline on interference testing approved in 2005. Results: Paired t-test comparing different doses of bilirubin revealed that the concentration of 1 000 $\mu\text{mol/L}$ bilirubin negatively interfered the determination of ALP levels. The experiment designed with five different concentrations of bilirubin showed that bilirubin can exert negative interference on the measurement of ALP in a linear pattern. Conclusion: High concentration of bilirubin can cause false measurement of ALP levels, probably interfering with the clinical prognosis of liver diseases.

Keywords: Alkaline phosphatase, bilirubin, interference

Introduction

Alkaline phosphatase (ALP) is a routine laboratory test used to screen for liver disease and elevated levels are often the first indication of chronic hepatic lesions, which is also an important index for evaluating the severity and prognosis of liver illnesses [1]. Recent data also indicated that elevated serum alkaline phosphatase levels are associated with increased mortality in patients with metabolic syndrome and they also suggested that higher levels of serum alkaline phosphatase is a predictor of mortality independent of the baseline prevalence of metabolic syndrome [2].

Hyperbilirubinemia is one of the common symptoms encountered by patients with liver diseases. A variety of previous investigations have proven that high concentrations of bilirubin exert significant interference on the determination of multiple biochemical substances [3-5].

This paper is designed to investigate whether high levels of bilirubin are able to pose interference upon the detection of ALP in patients with hyperbilirubinemia in laboratory settings, there-

by affecting the clinical diagnosis and prognosis of hepatic diseases in clinical practice. The laboratory procedures of the interference test were conducted strictly according to the Clinical and Laboratory Standards Institute (CLSI) document EP7-A2, the most recent guideline on interference testing approved in 2005 by the U.S. Committee for Clinical Laboratory Standards [6].

Materials and methods

Instruments and reagents

Instruments

Olympus AU5400 analyzer (Olympus America, Melville, NY). It has been verified that the linear range of total bilirubin is: 0-1009 $\mu\text{mol/L}$.

Reagents

The purity of standard bilirubin was 99%. The ALP activity was measured using an ALP kit based upon AMP buffer method. The levels of total bilirubin kits were measured by total bilirubin kits by employing the Diazonium method.

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Table 1. Number of replicates corresponding to d_{\max}/S

d_{\max}/S	No. of replicates	d_{\max}/S	No. of replicates
0.8	41	1.5	12
1	26	1.6	10
1.1	22	1.8	8
1.2	18	2	7
1.3	16	2.5	5
1.4	14	3	3

Note: where (within-run) standard deviations were calculated from 20 times repeated measurements of the control samples; d_{\max} is the maximum allowable interference when measuring concentration.

Methods

The end-point method was performed for the measurement of ALP and total bilirubin on the biochemical analyzer.

Preparation of bilirubin stock solution

A total of 60 mg bilirubin was accurately weighed and added into a test tube. Then 5 mL of 0.4% NaOH solution was supplemented and constantly shaken for even mixture. When approaching the presence of complete dissolution of bilirubin, 0.4% NaOH solution was supplemented by droplets until the presence of full dissolution. The saturated bilirubin solution was prepared and stored at -20°C freezer for subsequent preparation. After calculation, the concentration of bilirubin in the experiment was expected to be approximately 13 650 $\mu\text{mol/L}$. Considering the degradation property of bilirubin when placed under light, the final concentration of bilirubin in this experiment was determined as the mean value of 10 times measurements.

Preparation of basic samples

The concentration of the test specimens should be designed to include high, low and medical decision levels. Fresh serum was obtained from healthy individuals who had not taken medication in the past 3 days. The serum samples should have no signs of hemolysis, jaundice or lipemia.

Since bilirubin is an endogenous metabolite, therefore, the final concentration of bilirubin may be excessively high, which probably influences the credibility of the experimental out-

comes. To properly prevent the incidence of such events, the concentration of bilirubin in the basic samples was lower than 10 $\mu\text{mol/L}$. The final concentrations of ALP and bilirubin in the mixed serum were determined by laboratory instruments.

The respective serum samples with high and low levels of ALP were chosen and mixed with a rational proportion to adjust the final concentration of ALP up to 50, 150 and 400 U/L strictly in accordance with the medical decision level proposed by Stander et al.

Preparation of the control and test samples

Based upon the 3-year clinical experience in our hospital, the limit of high concentration of bilirubin was approximately 1 000 $\mu\text{mol/L}$. Therefore, the bilirubin level of the test samples was adjusted to 1 000 $\mu\text{mol/L}$. As required by document EP7-A2, the proportion of diluted sample should be controlled under 5%, suggesting that the concentration of interfering substances should be 20 times (or above) higher compared with the test samples. However, the maximal level of bilirubin of the test samples (saturation samples) did not exceed 13 650 $\mu\text{mol/L}$, significantly lower than the required level of 20 000 $\mu\text{mol/L}$. Thus, the volume of the undiluted solution was approximately 1/13 of the total volume and the sample concentration was diluted by approximately -7.7%.

Interfering sample preparation: 1200 μl basic sample + 100 μl stock solution of bilirubin.

Control sample preparation: 1200 μl basic sample + 100 μl 4% NaOH.

Number of replicates

The number of replicates was determined by initially calculating the ratio of the maximal allowable error (d_{\max}) and the within-run standard deviation (s) and then identified the number of replicates according to corresponding d_{\max}/s , as indicated in **Table 1**.

In this study, the maximum allowable error was determined based upon the biological variation, and the total error (TE) of ALP achieved 11.7% [1]. The d_{\max} of each item was equal to the means of the basic sample * the maximum allowable error %.

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Table 2. Preparation of dose-response sample formulation

Sequence No.	Formulation	Estimated concentrations of total bilirubin (μmol/L)
1 (L)	Low Pool 1300 μL	< 10
2 (3L + 1H)	Low Pool 300 uL+ High Pool 100 μL	250
3 (2L + 2H)	Low Pool 200 μL+ High Pool 200 μL	500
4 (1L + 3H)	Low Pool 100 μL + High Pool 300 μL	750
5 (H)	High Pool 1300 μL	1000

Table 3. Evaluation of the interfering effects of total bilirubin on the determination of ALP

Level	Base pool (mmol/L)	Total allowable error (TE%)	d_{max}	Within SD (S)	d_{max}/S	Number of replicates	Control pool (U/L)	Test pool (U/L)	dobs	dc	Interference judgment
LOW	53	11.70%	6.20	0.52	11.93	3	49.5	13.1	-36.4	0.49	Yes
MED	165.6	11.70%	19.38	1.06	18.28	3	152.2	118.4	-33.8	1.01	Yes
HIGH	433.3	11.70%	50.70	3.41	14.87	3	396.5	369.3	-27.2	3.24	Yes

Table 4. Interference effects of high concentrations of bilirubin on the measurement of ALP levels

	Control sample (U/L)	Test sample (U/L)	Interference effects error	Imprecision	Bias	Total allowable error
LOW	49.5	13.1	-73.54%	3.20%	6.40%	11.70%
MED	152.2	118.4	-22.21%			
HIGH	396.5	369.3	-6.86%			

Sample analysis

The test (T) and control (C) samples were analyzed in an alternating order, e.g. C1T1C2-T2C3T3....CnTn.

To prevent the system affected by the test samples, deionized water was additionally supplemented to protect the control samples influenced by the test samples. As shown in the formula C1T1Cx Cx C2T2Cx Cx C3T3...Cx Cx CnTn, the results of the additional control sample (Cx) were eliminated. The concentrations of bilirubin and ALP were detected simultaneously.

Data analysis

The obtained data were subject to “point estimation” in terms of the interfering effect by comparing the d_{obs} (defined as the difference between the average values of the test and control samples) and the cut-off value (d_c) using the following equation, $d_{obs} = \bar{x}_{test} - \bar{x}_{control}$. The interfering effect existed when $d_{obs} > d_c$.

Note: n is the actual sample size.

The cut-off value (d_c) can be computed for a two-sided test using the following equation:

$$d_c = (d_{null} + sZ_{1-\alpha/2}) / \sqrt{n}$$

Note: d_{null} was the value stated in the null hypothesis, constantly equal to 0.

The 95% confidence interval (CL) of the interfering effect can be calculated according to the following equation

$$95\% \text{ Confidence Interval (CL)} = (\bar{x}_{test} - \bar{x}_{control}) \pm t_{0.975, n-1} \sqrt{\frac{2s^2}{n}}$$

Note: s is within-run standard deviation, n is the number of replicates for each sample, $t_{0.975, n-1}$ is taken from a Student t-table as the 97.5th percentile of t -distribution with $n-1$ degrees of freedom. (For $n > 30$, substituting 2.0 for $t_{0.975, n-1}$ is a reasonable approximation).

Preparation of dose-response samples

The dose-response experiment determines the relationship between the interfering concentration and the magnitude of interference, which permits the estimation of the effect at any interfering concentration within the range tested, as illustrated in **Table 2**.

The low concentration samples can be prepared by referring to the preparation method for the control samples; The high concentration samples can be prepared according to the procedures for the test samples.

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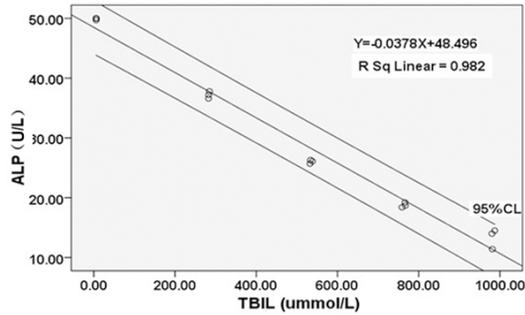


Figure 1. Dose-response experiment of bilirubin interference (low ALP).

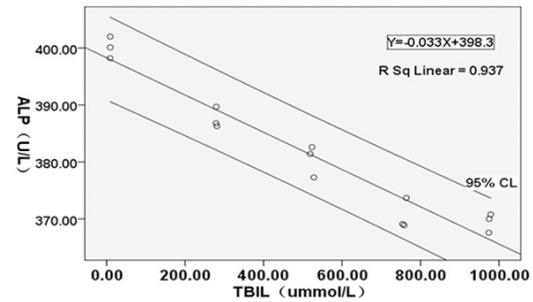


Figure 3. Dose-response experiment of bilirubin interference (high ALP).

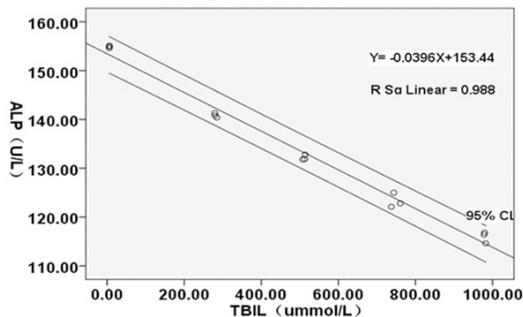


Figure 2. Dose-response experiment of bilirubin interference (medium ALP).

All samples were analyzed in a random order to avoid run-to-run variables

A total of five concentrations were analyzed within the same analytical run. The first set of replicates was analyzed in an ascending order, the second set in a descending order and the third set in an ascending order, etc., with the purpose to average out any systematic drift effects [2].

SPSS16.0 statistical software

SPSS16.0 statistical software was utilized for data analysis.

Results

Evaluation of the interference effects of total bilirubin was illustrated in **Table 3**.

The interference effects of high concentrations of bilirubin on the detection of ALP levels were shown in **Table 4** below.

Dose-response experiment of bilirubin interference ALP.

Evaluation of interfering effects

Different concentrations of interfering errors can be estimated by linear regression analysis (**Figures 1-3**).

Discussion

New protocols for measuring interfering substances are needed. These could be developed by a Clinical and Laboratory Standards Institute (CLSI) committee on interfering substances. The most recent CLSI guideline on interference testing in clinical (document EP7-A2) was approved in 2005 [6]. The testing of both interfering substances as well as analytical accuracy might be best performed by a notified body instead of the monitor's manufacturer. The evaluation scheme can be widely used in clinical laboratory testing methods, instruments and various types of specimens, especially for analyzing the potential interfering substances. In this study, "paired-difference" experiments at higher concentrations of potential interfering substances (bilirubin) for initial screening. After verifying that bilirubin is the interfering substance, "dose-response" experiments were conducted to evaluate the association between the concentration of interfering substances and the degree of interference. Whether the interfering effects of bilirubin upon ALP level was equally evaluated objectively.

As shown in **Table 4**, high concentrations of bilirubin played a role in the negative interference of ALP levels from low- and moderate-concentration samples were -73.54% and -22.21%, respectively, which exceeded the total allowable error of 11.7%. In this experiment, the outcomes and regression formulas proved that the interfering effects caused by different concentrations of bilirubin can be estimated.

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These results suggest that when patients were clinically diagnosed with hyperbilirubinemia, ALP alone should not be directly utilized as a diagnostic parameter if the interfering effects of bilirubin can not be confirmed. Bilirubin probably inhibits the enzyme activity, especially by exerting negative interfering effects since bilirubin is a type of reducing agent, which is able to weaken electron and proton transmission [7].

This study indeed has certain limitations as below: First, the substance of bilirubin supplemented to the serum samples may differ from the endogenous bilirubin yielded within human body. Second, the test samples used in this experiment are unable to represent the samples in clinical settings. Third, the concentration of the test samples may be excessively low, high.

Acknowledgements

The ethics committee of Beijing You'an Hospital approved this study.

Disclosure of conflict of interest

None.

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