

Stability of values for the activities of critical enzymes assayed in serum

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Abstract

Background: Assays for the activities of a set of enzymes in serum, *i.e.*, alkaline phosphatase (ALP), alanine amino transferase (ALT), aspartate amino transferase (AST), creatine phosphokinase (CK), and lactate dehydrogenase (LD) are critical in the diagnosis of a wide range of diseases. In view of reports that storage of some of these enzymes at 4°C or lower results either in progressive irreversible enzyme inactivation (CK and LDH) or activation (ALP), and because re-assays for these enzymes are requested, we have undertaken to study the stabilities of these enzyme activities over time (6 days) at 4°C.

Methods: Thirty patient samples, that were assayed for each of these enzyme activities and were stored at 4°C, were re-assayed on a Beckman-Coulter AU5800 analyzer. The mean values, standard deviations and coefficients of variation (CVs) were determined for each analyte in each sample over this time period. ANOVA analysis was performed for day-to-day variances for all samples to determine if the values obtained over the six day period were statistically the same.

Results: The average CVs for ALP, ALT, AST, CK and LD were, respectively, 1.98, 3.88, 3.88, 2.18 and 2.60. ANOVA analysis for the day-to-day variances of all sample values showed that these were the same over the six day period ($p>0.05$).

Conclusions: We conclude that the activities of these enzymes are stable at 4°C over the storage period of six days indicating repeat testing on patient samples will yield reliable results.

Introduction

Diagnosis of a wide range of diseases often depends on the values of particular activities of critical enzymes assayed in the sera of patients. These determinations enable diagnosis of possible hepatic (AST, ALT, LDH), biliary tract (ALP), skeletal muscle (CK) and, on occasion, cardiovascular (CK, AST, LDH) diseases. Elevations of any of these enzymes either as isolated values or some combination of elevated enzyme activity values point to abnormal conditions in specific tissues. Not infrequently, clinical laboratories are requested to repeat determinations of specific enzyme activities in cases where the values do not match the clinical impression. Generally, these requests must be made over the time course of no longer than 6-7 days, since samples are discarded, subsequent to analysis, after this time period.

In several prior studies, we examined the stabilities of a number of serum analytes included electrolytes [1], glucose [2], BUN [3] and creatinine [3,4]. These studies were prompted by specific findings such as slow increases in sodium values on the same samples after ion selective electrode replacement [1], effects of storage of samples prior to analysis [5] and random error in borderline values of creatinine [4]. In view of several recent requests for repeat analysis of several serum enzyme levels, we have extended our studies as part of our quality assurance program to include these critical enzymes.

This undertaking was further prompted by prior reports on the stabilities of these enzymes in serum. It has been reported that LDH, especially the LD5 isozyme, becomes irreversibly inactivated at refrigeration temperatures (4°C) causing significant decreases in its activity [6,7]. This finding was likewise reported in a study of multiple serum analytes incubated for one week at 9°C [8]. The stabilities of

ALP, ALT, AST and CK were found to occur within defined inaccuracy guidelines; only LDH was found to have serum values that exceeded these limits [8]. On the other hand, there have been additional reports that ALP may become activated over time especially in the presence of magnesium ions [6], and an on-line statement (Labpedia.net) concerning ALP stability is that at 0-4°C the assay values for this enzyme are stable only for 2-3 days. In an extensive study on the stability of analytes in rat sera, statistically significant decreases in the mean values of both CK and LDH were found on day 7 while mean values for ALP, ALT and AST were found to be stable [9]. In addition, it is known that ALT, a marker for acute liver disease including hepatitis, may be only marginally elevated in patients with hepatitis C, necessitating stable enzyme levels to be present in the event of re-assay [10]. This issue is the so-called "borderline value problem" encountered in borderline values for such analytes as creatinine [4], wherein values obtained for the same sample can alternate between normal or high causing difficulty in interpreting the result.

We have therefore undertaken, in a quality assurance study, to determine if the activities of these critical enzymes are stable in serum samples that have been stored for six days.

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Key words: enzyme activities, reproducibility, coefficient of variation, storage temperature, ANOVA test on variances

Received: July 18, 2017; **Accepted:** August 10, 2017; **Published:** August 14, 2017

Methods

Sample size and selection

To compute a statistically valid number of samples for our study, we used the following formula for n , the required number of samples that is based on set levels of confidence and reliability:

$$n = \frac{\ln(1 - \text{confidence})}{\ln(\text{reliability})} \quad (1)$$

The confidence level is usually 95 percent. Reliability is the degree of precision. Since, as discussed in the Results section, our coefficients of variation were low, we set the reliability to 0.9. For this formula, the failure rate must also be considered. However, since all of our values lay within 2 standard deviations of the means, as shown in the Results section, we assumed that no points “failed.” Therefore, failure rate was not entered into this equation. For 95% confidence level and 0.9 reliability, $n = 28.4$. We used thirty patient samples for our study, slightly more than the statistically required number of samples.

We obtained patient samples that represented a wide range of values for each of the five enzymes, ALP, ALT, AST, CK and LDH, whose activities were assayed. All samples were selected on the day that they were analyzed for these enzymes. All of these 30 samples were stored at 4°C over a period of six days after initial analysis.

Chemical analysis and statistical evaluation

On each day, after the first analysis, all five enzymes were assayed on a Beckman-Coulter AU5800 analyzer (Brea, CA). All analyses were performed only after the appropriate controls (a normal and a high level) (Biorad, Hercules, CA) were analyzed and whose values were found to be within plus or minus two [2] standard deviations from the determined mean for each enzyme activity assayed.

For each patient sample, for each enzyme assayed, the mean value and the standard deviation were computed. The precision, was computed as the coefficient of variation (CV), that was the standard deviation divided by the mean. The values for each enzyme for each patient were also subjected two tests for sameness: one was whether the values all lay within plus or minus two standard deviations of the mean, assuming a Gaussian distribution of values and a 95 percent confidence interval. The second test was a one-way ANOVA F-test on the day-to-day variances of the values of all thirty samples using an alpha level of 0.05. P values higher than this alpha level indicated no significant difference between values while p values less than this alpha level indicated significant differences.

Cumulative values for the coefficients of variation for each enzyme assayed were plotted as percent of samples having CVs that were less than or equal to a given CV, and the value of the CV for which 90 percent of the CV values were less than or equal to this value was determined.

Results

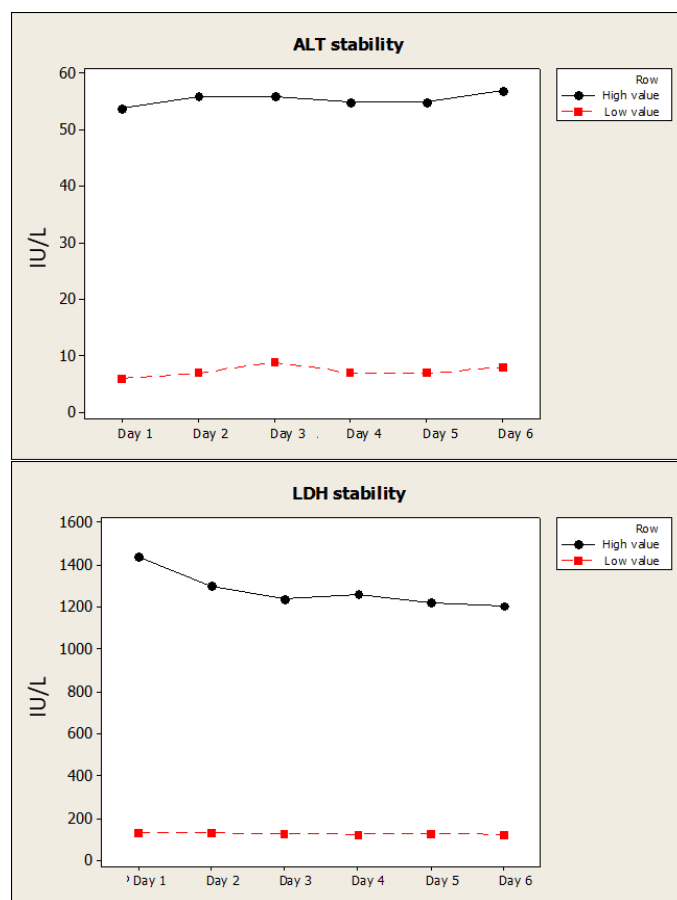
Mean CVs Are Low. Table 1 summarizes the results obtained for each of the five critical enzymes. As can be seen from this table, the mean coefficients of variation (CVs) for each enzyme assayed were low, all of them being lower than 4 percent. These CVs were obtained on sample values that covered a broad range and included elevated levels of each enzyme and samples with normal and low normal values as can be seen in the second column of Table 1. As can further be seen in the last column of Table 1, 90 percent of all CV values for all five enzymes were less than a maximum of 7.4 percent (for AST), indicative of acceptable CV values for each analyte.

Stabilities of individual samples

Occasional CV values were obtained that were significantly higher than the mean such as 14.08 and 12.06 for ALT and AST, respectively (column 4, entries 2 and 3 in Table 1). This observation reflects a phenomenon that occurs frequently in studies on the reproducibility of values [3, 4] higher values of CVs often are found for values that have low means. The ALT sample having the CV value of 14.08 had the following six values determined over the study's time course: 6, 7, 9, 7, 7, 8 with a mean of 7.33 and a standard deviation of 1.03. Although the CV is technically high, these values are reproducible, as shown in the upper panel in Figure 1, and the small differences between them have no clinical significance. Similar results were obtained on the AST sample having a CV of 12.06.

Also shown in the upper panel of Figure 1 is the plot of ALT values for a sample that lay slightly above the upper reference range value of 52 IU/L. The ALT activity values for this sample ranged from 54-57, i.e., the values for ALT were reproducibly slightly elevated indicating the possibility of hepatitis.

Figure 1 (lower panel) also shows examples of the reproducibility of LDH assays for low normal and elevated values. The low normal values (red filled squares in the lower panel of Figure 1) are very close to one another; the CV was 3.75. For the elevated values (black filled circles), there was a discernible decrease, by over 15 percent (1438 IU/L to 1210 IU/L), in the assay values from day 1-day 6. Nonetheless, the



Figures 1. Plots of high (black filled circles) and normal (red filled squares) activity values (IU/L on Y-axis for each plot) over the six day storage period for serum samples assayed for ALT (upper panel) and LDH (lower panel).

Table 1. Mean CV values for each critical enzyme (30 samples) together with their ranges and the ranges of values for each enzyme.

ENZYME ¹	RANGE OF VALUES (IU/L) ²	MEAN CV(%)	RANGE OF CVS	90% OF ALL CV VALUES (%) WERE ≤ THIS VALUE:
ALP	53-355 (34-104)	1.98	1.26-3.19	2.7
ALT	5-78 (7-52)	3.88	0-14.08	6.7
AST	13-130 (13-39)	3.88	0-12.06	7.4
CK	17-1305 (30-223)	2.18	0.97-7.16	4.6
LDH	125-1438 (140-271)	2.60	1.12-6.57	4.1

¹Abbreviations are: ALP, alkaline phosphatase; ALT, alanine amino transferase; AST, aspartate amino transferase; CK, creatine phosphokinase; LDH, lactate dehydrogenase.

²Lowest and highest values for each of the 30 samples for each enzyme. The reference range for each enzyme in serum is given in parenthesis.

CV was 6.57.

In fact, for a majority of samples assayed for LDH activity, we noticed a decrease in LDH activity over the six day period that was on the order of 2-5 percent. Most of the activities of the other four enzymes were not observed to undergo similar decreases. Therefore, there may be a decrease in LDH activity over storage time that does not cause significantly elevated CV values.

Further testing for reproducibility of values

To test our results further for reproducibility, we determined if all values obtained for each enzyme activity in each sample were within the limits of random error for the values that were obtained for each sample. First, assuming a normal distribution of values for each analyte in each of the thirty samples, we determined if all of the values lay within the 95 percent confidence interval for the values, i.e., whether the values occurred within plus or minus two standard deviations from the mean. By this test, we found that all values for the activities for each enzyme in each of the thirty samples lay within this confidence interval.

We also applied the one way ANOVA (F-test) analysis to the day-to-day variances of all thirty samples for each enzyme. The results of this analysis are shown in Table 2. For each enzyme assayed, the F score (column 3) and the p value for this F score (column 4) are given. Since all values for p were >0.05, indicating that the values are indistinguishable, we conclude that the values for all five enzymes did not change significantly over the storage period. Furthermore, the p values in Table 2 are 1.0 or close to 1.0, suggesting that an increase in sample size would not impact this finding.

Discussion

This study was undertaken to determine whether it was valid to perform repeat serum enzyme determinations on requests from the clinical staff during their six day storage period at refrigerator temperature (4°C). There are few systematic studies on the stabilities of human serum analytes at refrigeration temperatures, and some of the results of these studies are contradictory [8]. In addition, conditions are not always the same, creating a basis for differences in results. Our study is focused on the reproducibility of critical enzyme assay values in samples stored at refrigeration temperatures for six days.

As can be seen from the low values for the CVs for each enzyme as shown in Table 1 and the plots of typical values determined for stored samples both for high values of enzyme activities and normal values for these enzymes, the reproducibility of the values is satisfactory as shown in Figures 1. This conclusion has been further confirmed in our statistical analysis of the data which suggests that the activity values over time found for each enzyme are statistically indistinguishable from one another. We showed via ANOVA analysis that the values over the 6 day time period did not change with any statistical significance for all 30 samples.

Table 2. Statistical analysis of enzyme activity variations over time.

Enzyme	Average within subject coefficient of variation	F-score ¹	p-value ²
ALP	1.9813	0.03	1.000
ALT	3.8769	0.01	1.000
AST	3.8812	0.02	0.979
CK	2.1828	0.35	0.884
LDH	2.6097	0.37	0.867

¹F-test for significance of changes in the day-to-day variances of all values for each enzyme.

²p values > 0.05 are considered to indicate no statistical difference between the variations in the sample results between different days.

Since these results were obtained for samples that had a wide range of values for each enzyme, we further conclude that factors that can affect the activities over the storage time do not cause significant changes in activity over the six day time span.

Conclusion

Based on our findings that the CV values for each of the five critical enzymes in the thirty serum samples on which our assays were performed were low and that the results of the statistical analyses confirm reproducibility, we conclude that reproducible serum levels for these enzymes can be achieved at any time during their storage times.

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