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Importance of observing the progress curve during enzyme assay in an automated clinical chemistry analyzer: a case study

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ABSTRACT

In the case presented here the reported result for total creatine phosphokinase led to the physician calling for report confirmation. The repeated test result was in keeping with the clinical picture and thus the previous erroneous result was amended. The incorrect result from auto analyzer was identified as failure to run the sample in dilution after instrumental flagging of possible substrate exhaustion evidenced by erroneous progress curve. A frequent reason for nonlinear progress curves is the presence of excess enzyme which can be easily misinterpreted as lower enzyme activity in a provided sample. Careful inspection of progress curve and predilution of sample in anticipated cases could avoid erroneous result.

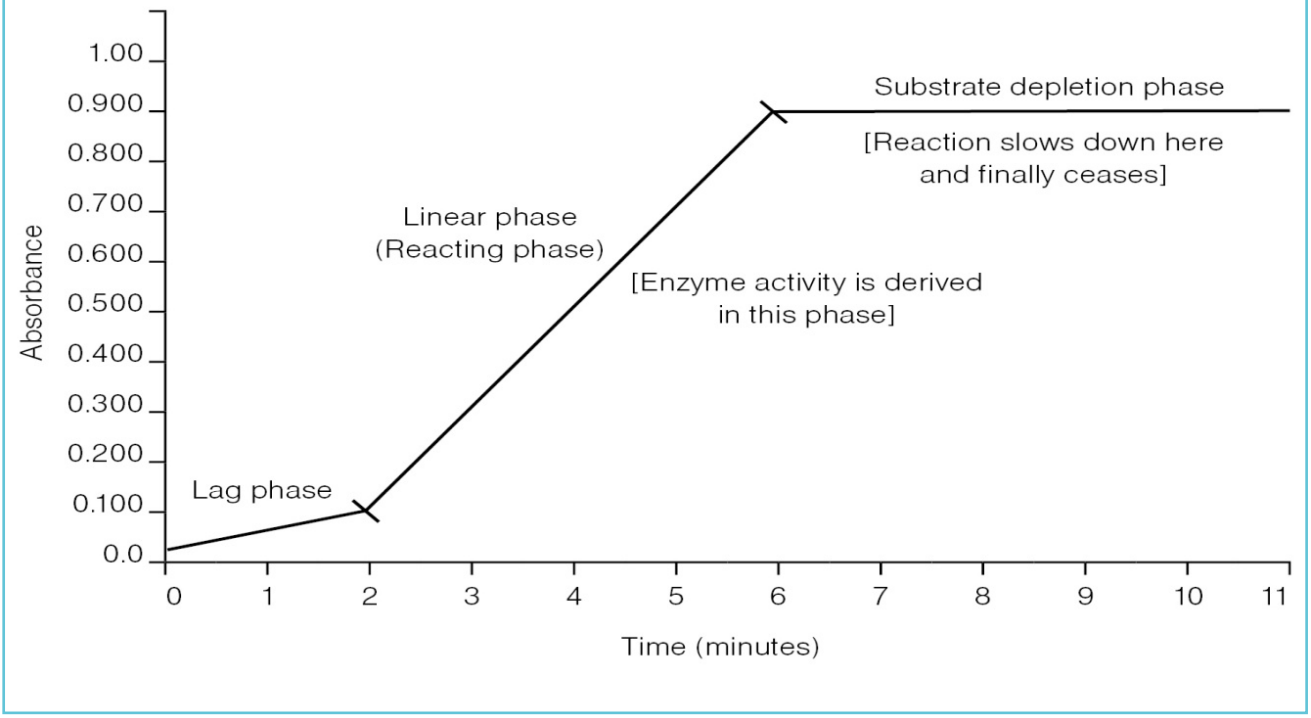
INTRODUCTION

Concentration of enzymes is very low in plasma or body fluid and cannot be directly measured as in case of other analytes such as glucose or total protein. Thus, enzymes are indirectly measured by their catalytic activity which is proportional to their concentration.

The principal of kinetic assay is that, if the concentration of the substrate (S) is sufficiently high in comparison to enzyme (E) then the rate of reaction will be proportional to the concentration of the enzyme. Therefore, the amount of product (P) formed in a given period of time would be proportional to the amount of active enzyme present, with all other factors remaining constant. To determine the reaction velocity and for evaluating the enzyme activity, the plot of absorbance against time is required. This plot also known as progress curve permits the detection of erroneous influences and the control of the reaction course. (Figure 1) A catalyzed reaction must initially follow a linear relationship, from which its velocity and eventually enzyme activity is derived. Due to depletion of substrates during the later progression the reaction slows down and finally ceases. (Figure 1)

The Michaelis-Menten equation provides the "activity" of the enzyme (1). The rate of reaction when the enzyme is saturated with substrate is the maximum rate of reaction, Vmax. The relationship between rate of reaction and concentration of substrate depends on the affinity of the enzyme for its substrate. This is usually expressed as the Km (Michaelis constant) of the enzyme, an inverse measure of affinity. For practical purposes, Km is the concentration of substrate which permits the enzyme to achieve half Vmax. An enzyme with a high Km has a low affinity for its substrate, and requires a greater concentration of substrate to achieve Vmax. This means that the concentration of substrate must be high enough to ensure that the enzyme

Figure 1 Normal progress curve of a catalyzed reaction



is acting at Vmax (1). In practice, it is usual to use a concentration of substrate about 10 – 20 fold higher than the Km in order to determine the activity of an enzyme in a sample (2). Majority of enzymes have Km values in order of 10^{-5} to 10^{-3} mol/L.

The present report emphasizes the importance of observing the plot of absorbance against time to identify the linearity slope, while performing creatine phosphokinase (CK) enzyme assay.

CK is a large protein made up of two distinct polypeptide subunits, M and B (3). Three isoenzymes of CK are found in human tissue: CK-MM (skeletal muscle), CK-MB (cardiac muscle), and CK-BB (brain). CK catalyses the reversible transfer of energy-rich phosphate from creatine phosphate to adenosine diphosphate (ADP), thus forming adenosine triphosphate (ATP) that is utilized by muscle myofibrils during its contraction. The molecular structure of CK prevents it from being released from the host tissue into the bloodstream, except in muscle membrane injury. Serum concentrations of CK is therefore increased when muscle is damaged after strenuous physical activity, trauma, crush injury, myositis, muscular dystrophy, intramuscular injection, convulsions, myocardial infarction, malignant hyperthermia and drugs such as aminophyline and succinylcholine (4). CK measurement in serum is the gold standard test to detect and monitor skeletal muscle diseases and damage. Thus, it is essential to mention the clinical diagnosis while ordering this test.

CLINICAL-DIAGNOSTIC CASE

We received a serum sample from a 48 years male collected at a remote hospital, for analysis of total creatinine kinase. The sample was analyzed using RX Imola auto-analyzer (Randox Laboratories Ltd). Daily maintenance for this auto analyzer was conducted and internal quality control sample from Bio-Rad was run which were found to be within the acceptable range. The patient result of 12 U/L [24 – 195 U/L] was reported. However, we received a call from attending physician stating that they had expected higher value for total CK and requested to repeat the assay. The provisional diagnosis of patient was acute kidney injury secondary to lacerated wound over left lower limb.

Careful inspection for potential source of error in all analytical steps and reanalyzing the serum sample was planned.

Preanalytical factors affecting CK are age, race, muscle mass, physical activity, medication and hypothyroidism. Only severe hemolysis affects CK since red blood cells (RBC) have no CK. Release of enzymes and intermediates from RBC such as adenylate kinase (AK), ATP and glucose-6-phosphate (G-6-P) may inhibit CK in severe hemolysis. No preanalytical error was identified in the index case.

The reagent, calibrator, internal quality control graphs and instrument were checked to identify presence of any analytical error. Total CK is performed in serum or heparinised plasma. CK enzyme activity is easily inhibited by factors both in vivo and in vitro, thus automated assay includes factors to preserve enzyme action and ensure accurate measurement. For example, the reagent contains N-acetyl cysteine (NAC) to reactivate sulfhydryl group in the centre of CK which is prone to rapid oxidation with loss of CK activity. The diadenosine pentaphosphate and adenosine monophosphate (AMP) is added to inhibit adenylate kinase, which may be present in platelets of patient with liver disease. Also, the reagent contains magnesium to complex with ADP and ATP.

The enzymatic reaction catalyzed by CK is reversible, however all commercial assays for CK are based on creatine-to-creatine phosphate reaction as it proceeds six times faster than creatine phosphate to creatinine. ATP liberated

phosphorylates glucose to glucose-6-phosphate. Glucose-6- phosphate is oxidized to 6-phosphogluconate, reducing Nicotinamide adenine dinucleotide phosphate (NADP) to NADPH in presence of glucose 6-phosphate dehydrogenase (G6PD). The rate of increase in NADPH absorbance at 340nm is directly proportional to the activity of CK present in serum. The difference between commercial assays is the use of buffer (imidazole or Tris) and source of G6PD (yeast or from bacterium) that however does not interfere with the assay. The reagent was in standard condition and no error was identified during sampling.

The internal quality control for CK in the past ten days was within 1SD and calibration was up to date. There was no recent maintenance of instrument and no issues with other parameters. The technician was however recently employed. The reported result was inspected in the instrument, and it was found that the assay was repeated automatically with two results where the first one indicated error with flagging as "E" and second one was 12 U/L. Thus, the technician reported the result as 12 U/L through laboratory information system (LIS). The inspection of progress curve indicated an erroneous result.

The normal progress curve for each analyte could be inspected in the auto analyzer, from the stored data generated by sampling of the quality control for that specific analyte. Any deviation from this normal progress curve should alert the technician. Thus, analytical error was identified and repetition of the test from stored sample was done.

We anticipated higher value for total CK from the provisional diagnosis. Thus, we diluted the sample and the total CK was 150891.98 U/L after 1:100 dilutions. The result was informed to the physician and the initial erroneous result was amended.

DISCUSSION

The basic principle of kinetic enzyme assay is that when the substrate concentration is very high the reaction rate is independent of substrate concentration. (1) Under the normal circumstances, the concentration of the substrate contained in a reagent is large enough to meet the actual testing needs of most clinical samples. Enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST) and creatine kinase (CK) may be released in very large quantity in certain clinical conditions affecting liver, heart, muscle or other organs. During such a clinical situation, the enzyme activity exceeds the linear range of the kit which no longer maintains the reaction at zero order and the reported results are far below the actual concentration.

In practice the reaction rate is not constant with time. There is an initial (i) lag phase (with very little change per unit of time), then comes (ii) linear phase of constant change per unit of time and then finally (iii) phase of substrate exhaustion with very little change. In order to obtain the best result we should have a long linear phase and assay should be stopped before substrate exhaustion takes place. Too much of enzyme will consume all the substrate immediately and no more reaction can be observed when the recording is started. Such a situation is easily misinterpreted as lack of activity and the reported enzyme concentration is very low. The substrate in the above mentioned reaction is creatine phosphate. Creatine phosphate gets consumed by very high concentration of CK which was present in the serum sample before the kinetic measurement actually gets initiated. The whole substrate gets consumed within the lag phase which results in falsely low values.

This effect is also a recognized limitation in immunoassay, where it is known as prozone effect which occurs when the number of analyte

molecules exceeds the number of antibody binding sites. There are numerous reports of this effect in wide ranges of analytes as shown in Table 1. Many modern immunoassays have been formulated for automated dilution in cases of prozone effect.

The clinical performance of the biochemistry analyzer is affected by many factors such as the reagents, the integration and settings, the traceability system of the manufacturer, daily calibration and quality control operation and maintenance of the instrument. When choosing an analyzer, the laboratory should pay special attention to the substrate depletion limit of the kinetic method. Different laboratories may have different ranges of substrate depletion due to variable detection system, sample dilution ratios and parameter settings. Automatic dilution of sample in cases of substrate exhaustion was lacking in our analyzer and the recently employed technician failed to recognize the instrumental flagging for erroneous result which led to wrong reporting initially.

The erroneous result from auto analyzers could be avoided by observing the plot of absorbance against time to identify the linearity slope since various analyzers usually present this figure. Those instruments which display the absorbance and continue to display the changing absorbance with the chemical reaction are considered superior for kinetic assays. It can monitor the rate of reaction depending on the high or low concentration of enzyme and also help in detecting substrate exhaustion along with timings. If we can anticipate higher values of the enzyme (beyond the linearity range) we can predilute the sample and then run the test so as to avoid repetition. For example: an icteric sample may have higher values of transaminase and if clinical history of trauma or lacerated wound is provided, then the sample may have high CK value (Table 2). This case therefore also

Table 1 Reports of clinical assay demonstrating prozone effect

Biochemical assay	Clinical diagnosis	Reference
Prolactin	Prolactinoma	5,6
β-hCG	Advanced molar Pregnancy	7
Calcitonin	Metastatic medullary thyroid carcinoma	8
Prostate specific antigen	Advanced prostate cancer	9
17-hydroxyprogesterone	21-hydroxylase deficiency	10
CA-125	Ovarian carcinoma	11
Alpha fetoprotein	Hepatoblastoma	12
IgE	Atopic dermatitis	13

Table 2 Biochemical parameters where the reading of progress curves is important

Analyte	Suggestive history or clinical condition where predilution of sample is helpful
ALP	Icteric sample, Obstructive jaundice, Bone tumor
Amylase and Lipase	Lipemic sample, Patient under evaluation for pain abdomen, History of alcohol intake
CK	Muscle trauma, Crush Injury, muscular dystrophy
CK-MB	Electrocardiogram changes, Myocardial infarction
ALT	Icteric sample, Liver disease
AST	Icteric sample, Liver disease
Gamma GT	Icteric sample, Liver disease
LDH	Cancer, Anemia, Myocardial infarction
Myoglobin	Muscle trauma, Rhabdomyolysis

highlights the importance of mentioning the provisional diagnosis while ordering the test.

LEARNING POINTS

- A frequent reason for nonlinear progress curves is the presence of excess high enzyme level which is easily misinterpreted as lack of enzyme activity in the reported result.
- Careful inspection of progress curve and predilution of sample in anticipated cases could avoid erroneous result.

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