Assay Optimization of Lactate Levels in Athletes Using Nanophotometry Methods

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Abstract. Lactate levels are the main parameter used in sports as a marker of fatigue. In general, the measurement of lactate levels in research uses the rapid test diagnostic test method. Biochemically there will be a difference between rapid test measurements with enzymatic methods. But there are many factors to consider in enzymatic testing. The purpose of this study is to optimize the substrate volume and total reaction volume in enzymatic measurement of lactate levels using a nanophotometer. Lactate can be detected in biological samples such as serum, red blood cells, cell culture, and fermentation media. In this study serum was used as a sample obtained from a university student who carried out swimming activities as far as 200m. Then the serum is used to measure lactate levels using the Lactate Assay Kit (MAK064, Sigma Aldrich). Lactate levels were read calorimetric at a wavelength of 570nm with a nanophotometer. Optimization results show the optimum substrate volume is 1.5 μl with 11 μl buffer with the final reaction volume of 25 μl. Standard curves showed regression values (R²) 0,9909, and lactate levels in2,848nmol/μl samples.

1. Introduction

Physical exercise is an activity that is carried out repeatedly and continuously. Excessive physical exercise will result in an increase in metabolism in the body. The increase in metabolism that occurs in the body will result in the formation of lactic acid. Excessive formation of lactic acid will result in a buildup of lactic acid in the blood and in the muscles, which will result in body fatigue. Lactic acid is the end product of one of the energy pathways in the body known as glycolysis \(^1\). Agrawal et al., (2004) stated that lactate levels can be measured in plasma, serum, or blood.

The method of measuring lactic acid can be done enzymatically. Enzymatic measurements have certain characteristics that require enzymes in the reaction, the presence of enzymes can speed up the reaction. Enzymes also work specifically, and in enzymatic measurements, only lactic acid was measured. In enzymatic measurement, the sample size is optimized first to obtain valid and accountable data. A standard sample that can be used is about 0.5-10 μL per test. Many factors must be considered during the optimization of enzyme tests, namely the choice of buffer and its composition, the type of enzyme and its concentration, as well as the type of substrate and concentration, reaction conditions, and the appropriate testing technology \(^2\).

According to Sudjarwo (2001) in biological samples, analyte levels are generally very low and biological samples themselves contain many components or complexes \(^3\). At very low levels, there will be a large error. Especially in biological samples that contain lots of components. To reduce the
errors that occur, optimization steps are needed. And optimization also aims to determine the optimum sample volume in the enzymatic measurement of lactic acid.

This enzymatic optimization of lactic acid uses a nanophotometer. This nanophotometer works in the same way as a spectrophotometer. A spectrophotometer is a tool used to measure absorbance by passing light with a certain wavelength on a glass or quartz object called a cuvette \[4\]. One of the weaknesses of a spectrophotometer is that the final volume of the reaction is quite large when compared to a nanophotometer which only requires about 2-3 \(\mu\)L of volume. Based on the measurement procedure of the lactate assay kit the final reaction volume was only 100 \(\mu\)L. Therefore, the variation of the reaction volume is carried out in the use of a nanophotometer.

Based on the description above, a study was carried out that would optimize the volume of blood serum samples in enzymatically measuring lactic acid using a nanophotometer.

2. Materials and Method

2.1. Equipment

Centrifuge of blood was obtained by Labnet Prism R, Refrigerated Microcentrifuge. UV-Vis spectroscopy devices was obtained by Implen N60 UV-Vis Nanophotometer.

2.2. Requirements

Protective mask, Gloves, Beaker, Microtesttube, 96 well flat-bottom plate. Micropipete, Light Yellow Vacutainer blood collection tube.

2.3 Materials

Required Material to prepare lactate assay, Serum, Lactate Assay Kit (MAK064, Sigma Aldrich) contains Lactate Assay Buffer, Lactate Probe, in DMSO, Lactate Enzyme Mix, L(+) Lactate Standard, 100 nmole/mL.

2.4 Methods

2.4.1. Lactate Standards Preparation. Dilute 10 \(\mu\)L of the 100 nmole/\(\mu\)L Lactate standard with 990 mL of Lactate Assay Buffer to generate a 1 nmole/\(\mu\)L standard solution. Add 0, 2, 4, 6, 8, and 10 \(\mu\)L of the 1 nmole/\(\mu\)L Lactate standard into a 96 well plate, generating 0 (blank), 2, 4, 6, 8, and 10 nmole/well standards. Add Lactate Assay Buffer to each well to bring the volume to 50 \(\mu\)L.

2.4.2. Master Mix Preparation. First, for full reaction add in a microtube 46 \(\mu\)L Lactate Assay Buffer, 2 \(\mu\)L Lactate Probe, 2 \(\mu\)L Lactate Enzyme Mix for each well. Second, for half reaction add in a microtube 23 \(\mu\)L Lactate Assay Buffer, 1 \(\mu\)L Lactate Probe, 1 \(\mu\)L Lactate Enzyme Mix for each well. Third, for ¼ reaction add in a microtube 11.5 \(\mu\)L Lactate Assay Buffer, 0.5 \(\mu\)L Lactate Probe, 0.5 \(\mu\)L Lactate Enzyme Mix for each well.

2.4.3. Sample Preparation. In this research we were investigating optimum volume of sample and optimum final volume of reaction. Serum samples (0.5-10 \(\mu\)L/assay) can be assayed directly by adding in duplicate to 96 well plate based on manual kit instruction. We used 9 gradient of serum volume to be 0.5; 1; 2; 3; 5; 10 \(\mu\)L. Add Lactate Assay Buffer to each well to bring the volume to 50 \(\mu\)L for full reaction, to 25 \(\mu\)L for half reaction, and 12.5 \(\mu\)L for ¼ reaction.

2.4.4. The Lactate assay reaction. First, for full reaction added 50 \(\mu\)L of Lactate Standard (0, 2, 4, 6, 8, and 10 nmole/\(\mu\)L) into a 96 wellplate, added 50 \(\mu\)L of serum sample and add 50 \(\mu\)L of Master the
Reaction Mix to each of the wells. For half reaction add 25µL of Lactate Standard (0, 2, 4, 6, 8, and 10 nmole/µL) into a 96 well plate, add 25µL of serum sample too into other well plate and added 25µL of Master the Reaction Mix to each of the wells. For ¼ reaction added 12.5µL of Lactate Standard (0, 2, 4, 6, 8, and 10 nmole/µL) into a 96 well plate, added 12.5µL of serum sample too into other well plate and added 12.5µL of Master the Reaction Mix to each of the wells. Mix all well using a horizontal shaker or by pipetting, and incubate the reaction for 30 minutes at room temperature. Protect the plate from light during the incubation. And measured the absorbance at 570 nm (A570).

The absorbance was measured by Implen N60 UV-Vis Nanophotometer. The volume of sample was used in 1-3µL. All of sample and standard was obtained in duplo.

2.4.5. The method of data analysis. We made a standard curve from absorbance of lactate standard (0, 2, 4, 6, 8, and 10 nmole/µL). The values obtained from the appropriate lactate standards to plot a standard curve was used to be determined the amount of lactate present in the samples. The concentration of lactate in sample was calculation with equation

\[ C = \frac{S_a}{S_v} \]  

\( S_a = \) Amount of lactate acid in unknown sample (nmole) from standard curve  
\( S_v = \) Sample volume (mL) added into the wells.  
\( C = \) Concentration of lactate acid in sample  
Lactate molecular weight: 89.07 g/mole

3. Result and Discussion

From the research that has been carried out from the optimization of blood serum sample volume in enzymatic measurement of lactic acid levels using a nanophotometer, the following results are obtained.

![Figure 1: Standard curve of lactate](image)

Based on the standard curve, the linear regression value \( (R^2) \) is 0.9287 with the line equation \( Y = 0.6916x + 1.0024 \). From this equation the level of lactate in the blood \( (S_a) \) can be determined.

The final volume of the reaction consisted of three variations, namely full reaction (50 µL), half reaction (25 µL), and a quarter reaction (12.5 µL). The optimization of the measurement of lactate levels is done by varying the final volume and the volume of the sample used. The sample volume consisted of 0.5 µL, 1 µL, 1.5 µL, 2 µL. Lactate levels in the blood can be seen from tables 1, 2, and 3.

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### Table 1. Lactate levels full reaction

| Volume | Sample | Range of absorbance (A) | Lactate levels (y=0.6916x+1.0024) | Lactate concentrations (nmole/µL) |
|--------|--------|-------------------------|-----------------------------------|----------------------------------|
| 0.5 µL | A1     | 3.289                   | 3.306                             | 6.612                            |
| 1 µL   |        | 3.969                   | 4.289                             | 4.289                            |
| 1.5 µL |        | 5.309                   | 6.227                             | 4.151                            |
| 2 µL   |        | 4.983                   | 5.756                             | 2.878                            |
| 0.5 µL | A2     | 2.200                   | 1.732                             | 3.463                            |
| 1 µL   |        | 4.119                   | 4.506                             | 4.506                            |
| 1.5 µL |        | 5.916                   | 7.105                             | 4.736                            |
| 2 µL   |        | 5.974                   | 7.189                             | 3.594                            |
| 0.5 µL | A3     | 3.245                   | 3.243                             | 6.485                            |
| 1 µL   |        | 4.341                   | 4.827                             | 4.827                            |
| 1.5 µL |        | 3.904                   | 4.195                             | 2.797                            |
| 2 µL   |        | 5.463                   | 6.450                             | 3.225                            |
| 0.5 µL | A4     | 0.974                   | -0.041                            | -0.082                           |
| 1 µL   |        | 4.543                   | 5.119                             | 5.119                            |
| 1.5 µL |        | 3.529                   | 3.653                             | 2.436                            |
| 2 µL   |        | 5.699                   | 6.791                             | 3.395                            |

### Table 2. Lactate levels half reaction

| Volume | Sample | Range of absorbance (A) | Lactate levels (y=0.6916x+1.0024) | Lactate concentrations (nmole/µL) |
|--------|--------|-------------------------|-----------------------------------|----------------------------------|
| 0.5 µL | A1     | 0.955                   | -0.069                            | -0.137                           |
| 1 µL   |        | 5.045                   | 5.845                             | 5.845                            |
| 1.5 µL |        | 5.459                   | 6.444                             | 4.296                            |
| 2 µL   |        | 4.064                   | 4.427                             | 2.213                            |
| 0.5 µL | A2     | 1.130                   | 0.184                             | 0.369                            |
| 1 µL   |        | 5.873                   | 7.043                             | 7.043                            |
| 1.5 µL |        | 5.808                   | 6.949                             | 4.632                            |
| 2 µL   |        | 5.069                   | 5.880                             | 2.940                            |
| 0.5 µL | A3     | 3.713                   | 3.919                             | 7.839                            |
| 1 µL   |        | 3.901                   | 4.191                             | 4.191                            |
| 1.5 µL |        | 4.478                   | 5.025                             | 3.350                            |
| 2 µL   |        | 6.184                   | 7.492                             | 3.746                            |
| 0.5 µL | A4     | 4.691                   | 5.333                             | 10.667                           |
| 1 µL   |        | 4.809                   | 5.504                             | 5.504                            |
| 1.5 µL |        | 4.120                   | 4.508                             | 3.005                            |
| 2 µL   |        | 4.664                   | 5.294                             | 2.647                            |
Table 3. Lactate levels quarter reaction

| Volume | Sample | Range of absorbance (A) | Lactate levels (y=0.6916x+1.0024) | Lactate concentrations (nmole/µL) |
|--------|--------|-------------------------|-----------------------------------|-----------------------------------|
| 0.5 µL | A1     | 0.670                   | -0.481                            | -0.961                            |
| 1 µL   |        | 3.133                   | 3.081                             | 3.081                             |
| 1.5 µL |        | 5.470                   | 6.460                             | 4.307                             |
| 2 µL   |        | 4.199                   | 4.622                             | 2.311                             |
| 0.5 µL | A2     | 0.624                   | -0.547                            | -1.094                            |
| 1 µL   |        | 3.883                   | 4.165                             | 4.165                             |
| 1.5 µL |        | 5.804                   | 6.943                             | 4.628                             |
| 2 µL   |        | 7.071                   | 8.775                             | 4.387                             |
| 0.5 µL | A3     | 3.512                   | 3.629                             | 7.257                             |
| 1 µL   |        | 4.328                   | 4.809                             | 4.809                             |
| 1.5 µL |        | 4.404                   | 4.918                             | 3.279                             |
| 2 µL   |        | 4.524                   | 5.092                             | 2.546                             |
| 0.5 µL | A4     | 6.143                   | 7.433                             | 14.866                            |
| 1 µL   |        | 4.006                   | 4.343                             | 4.343                             |
| 1.5 µL |        | 4.830                   | 5.534                             | 3.690                             |
| 2 µL   |        | 4.976                   | 5.746                             | 2.873                             |

Based on (Table 1) full reaction measurement, the results showed that the sample volume was 0.5 µL, 1 µL and 2 µL the absorbance was unstable, while at the sample volume of 1.5 µL the absorbance was stable. And in the measurement of lactate levels in the full reaction measurement sample there were results that showed negative (-) at a volume of 0.5 µL and at sample volumes of 1 µL, 1.5 µL and 2 µL there were low results. The low yield of lactate levels in the sample at the full reaction measurement was caused by the low volume of the input sample, while the final volume of the reaction was large, namely 50 µL.

Based on (Table 2) half-reaction measurements, the results showed that the sample volume of 0.5 µL, 1 µL and 2 µL of absorbance was unstable, while the sample volume of 1.5 µL the absorbance was stable. And in the measurement of lactate levels in the sample in the half-reaction measurement there were still negative results (-) on the sample volume of 0.5 µL and the sample volumes of 1 µL, 1.5 µL and 2 µL were low results but had increased from the full reaction measurement. This was due to a reduction in the final volume to 25 µL.

Based on (Table 4) measurement of a quarter reaction, the results showed that the sample volume of 0.5 µL, 1 µL and 2 µL of absorbance was unstable, while the sample volume of 1.5 µL of the absorbance was stable. And in measuring the levels of lactic acid in the sample in the measurement of a quarter reaction, there were still negative results (-) for the sample volume of 0.5 µL and the sample volumes of 1 µL, 1.5 µL and 2 µL the results obtained have increased from the half-reaction measurement. This resulted in a reduction in the final volume to 12.5 µL. According to Flora (2015) shows that anaerobic physical activity results in an increase in plasma lactate levels [5]. Tolerance of lactic acid levels in humans is estimated to be above 20 mM / l blood and 25 mM / k g wet muscle weight, and can even reach above 30 mM / l in dynamic training with high intensity [6]. In line with Nikseresht et al., (2017) the results of his study showed a significant increase in blood lactate after the test, and decreased to below baseline 24 hours after the test [7]. This is in accordance with Syahrastani’s research states that exercise intensity affect cellular adaptation to achieve homeostatic balance [8]. Besides that, the role of gene expression also helps in maintaining the balance of the body, one of which is the hif-α gene [9].
Based on the tables (1, 2, 3) it can be seen that the optimum measurement of lactic acid levels in the sample is a sample volume of 1.5 µL. This is due to the fact that in each full reaction measurement, half-reaction measurement, and measurement of a quarter of the reaction the results obtained at a sample volume of 1.5 µL remain stable / constant.

4. Conclusion
The optimum blood serum sample volume in measuring lactate acid levels is 1.5µL for a total volume of 12.5µL.

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