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Development of an enzymatic assay to measure lactate in perchloric acid-precipitated whole blood

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ABSTRACT

Background: The lactate to pyruvate (L:P) ratio is used to identify the cause of a lactic acidosis. Because tests for whole blood lactate and pyruvate require different sample types, the accuracy of the L:P ratio may be compromised by preanalytical errors. The measurement of lactate in the sample required for pyruvate is desirable.
Methods: Whole blood was added to 8% perchloric acid to obtain a protein-free supernatant. Lactate was measured by its oxidation to pyruvate and hydrogen peroxide by lactate oxidase. Assay accuracy, imprecision, analytical sensitivity, linearity, analyte stability, and a reference interval were determined.
Results: Deming regression of lactate results from paired plasma and supernatant produced a slope of 0.95 and *y*-intercept of −0.37 mmol/l ($R^2 = 0.95$). Recovery of lactate added to supernatant ranged from 103.4 to 112.7%. Within-laboratory CVs were 6.1% and 1.1% at 1.58 and 10.89 mmol/l, respectively and between-day CVs were 2.3% and 0.9%, respectively. The limit-of-detection was 0.18 mmol/l and the assay was linear to 13.15 mmol/l. Lactate in the supernatant was stable for a minimum of 8 h, 21 days, or 6 months at room temperature, 4–8 °C, and −20 °C, respectively. The lactate reference interval was 0.31–2.00 mmol/l from 116 healthy adults.
Conclusions: Lactate can be quantified in the same protein-free supernatant used for the measurement of pyruvate allowing the calculation of the L:P ratio from a single specimen.

1. Introduction

Pyruvate is a critical cellular metabolite derived from the oxidation of glucose during glycolysis and is subsequently metabolized by one of four different enzymes: 1) lactate dehydrogenase, resulting in the formation of lactate; 2) alanine aminotransferase, resulting in the production of alanine; 3) pyruvate carboxylase, resulting in the conversion of pyruvate to oxaloacetate; and 4) pyruvate dehydrogenase complex (PDC), resulting in the formation of acetyl-CoA that enters the tricarboxylic acid (TCA) cycle. The TCA cycle converts acetyl-CoA to carbon dioxide and energy via the production of reduced cofactors (NADH and $FADH_2$) that are subsequently used to generate ATP through the electron transport chain.

Disorders of pyruvate metabolism and the TCA cycle are clinically and genetically diverse but share many common manifestations including elevated concentrations of pyruvate and lactic acidosis [1]. The activity of PDC is integral to cellular energetics as it is the rate-limiting step of glycolysis. As such, deficiency of PDC activity, which impairs the mitochondrial oxidation of pyruvate and promotes its cytoplasmic reduction to lactate, is one of the most common congenital causes of impaired pyruvate metabolism in children [2].

The lactate to pyruvate (L:P) ratio is correlated to the cytoplasmic NADH:NAD^+ ratio (approximately 25:1) and is therefore a useful surrogate of the oxido-reduction state of the cytosol [2]. Clinically, the L:P ratio has been used to help identify the cause of lactic acidosis and to distinguish between PDC deficiency and other causes of congenital lactic acidosis [1,3,4]. Because PDC functions upstream of the TCA cycle, its deficiency does not alter the cytoplasmic oxido-reduction state and so the L:P ratio is low (<25) [2]. In contrast, inherited disorders of the respiratory chain complex or the TCA cycle increase the concentrations of NADH and $FADH_2$ and impair the oxidation of pyruvate leading to lactic acidosis and an L:P ratio >25 [2].

Conventionally, lactate is measured in plasma following the collection of whole blood into fluoride-oxalate blood collection tubes. Fluoride is a strong inhibitor of glycolysis and its presence prevents the formation of lactic acid after sample collection. Lactate measurements are also performed in heparinized whole blood by many blood gas analytical systems. In contrast, pyruvate is measured in a protein-free supernatant prepared by the addition of whole blood to perchloric acid which inactivates the enzymes that metabolize pyruvate [5]. Subsequently,

Abbreviations: L:P, lactate to pyruvate; PDC, pyruvate dehydrogenase complex; TCA, tricarboxylic acid cycle.

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calculating the L:P ratio requires the use of 2 different sample types that are collected and processed separately which raises concerns regarding preanalytical sources of error. Utilizing a single specimen for both pyruvate and lactate measurements is desirable.

2. Materials and methods

Unless otherwise specified, samples were prepared by adding 1 ml of whole blood anti-coagulated with heparin or EDTA to pre-chilled tubes containing 2 ml of 8% (w/v) perchloric acid [6,7], well-mixed for 30 s, incubated on ice for 10 min, and then centrifuged at 1500 ×*g* for 10 min to obtain a protein-free supernatant.

Lactic acid measurements were performed on a Roche cobas c501 chemistry analyzer (Roche Diagnostics) using a commercially available method (Trinity Biotech) in which lactic acid was converted to pyruvate and H_2O_2 by lactate oxidase. A peroxidase catalyzed the H_2O_2 formed in an oxidative condensation with a chromogen to produce a colored product with an absorption maximum at 540 nm. The increase in absorbance was directly proportional to the lactic acid concentration in the sample. The assay was calibrated using a 2-point calibration curve with 0 and 4.44 mmol/l lactate calibrators, the latter of which was diluted 1:3 with 8% perchloric acid prior to use. Prior to analysis, 120 μl of protein-free supernatants and calibrators were buffered by the addition of 30 μl of 1.5 mol/l Tris base.

The effects of sample processing, accuracy, imprecision, analytical sensitivity, linearity, and analyte stability were evaluated. Reference intervals were established from 116 healthy adults.

GraphPad Prism (ver 5.0f) and StatisPro, ver 2.51 (Clinical and Laboratory Standards Institute) were used for statistical analyses. As appropriate, continuous data were assessed by the nonparametric unpaired *t*-test (Mann–Whitney test), the nonparametric paired *t*-test (Wilcoxon matched-pairs signed rank test), or a nonparametric one-way ANOVA (Friedman test). The University of Utah Institutional Review Board approved the study.

3. Results

Three methods of processing whole blood with perchloric acid to obtain a protein-free supernatant were evaluated using 10 whole blood samples anti-coagulated with heparin collected from each of 10 volunteers. For the first method, blood was added to pre-chilled perchloric acid tubes, mixed, incubated on ice for 10 min, then centrifuged and the supernatant stored at −20 °C for 24 h. This method was regarded as the reference method as it is the process used for the preparation of samples for pyruvate testing in our laboratory. For the second method, blood was added to room temperature perchloric acid tubes, mixed, then centrifuged and the supernatant stored at −20 °C for 24 h. For the third method, blood was added to room temperature perchloric acid tubes, mixed, and the uncentrifuged sample stored at −20 °C for 24 h (these samples were centrifuged prior to testing to obtain supernatant). Compared to the reference method, failure to incubate samples on ice for 10 min after adding to perchloric acid or failure to immediately centrifuge after incubation significantly decreased lactate by 22% ($p = 0.002$) and 27%, respectively ($p = 0.004$) (Fig. 1).

To assess accuracy, 22 pairs of fluoride-oxalate and lithium heparin anti-coagulated whole blood were prepared from blood obtained from volunteers. Exogenous lactate was added to these pairs to create samples with lactate concentrations between 0.73 and 10.74 mmol/l. Immediately after the addition of lactate, the fluoride-oxalate anti-coagulated samples were centrifuged to obtain plasma and the heparinized anti-coagulated samples were processed to obtain protein-free supernatants. Lactate was measured in 2 replicates in both sample types. Deming regression produced a slope of 0.95 and an intercept of −0.37, and R^2 of 0.95 (Fig. 2). Accuracy was also evaluated by performing recovery experiments. Exogenous lactate was added to 2 aliquots each of EDTA and lithium heparin anti-coagulated whole blood to achieve 2 different target concentrations prior to preparing protein-free supernatants. Lactate was measured in 2 replicates. The mean ± SD lactate recovery was 110 ± 2.2% (range, 108–113%) and 106 ± 1.5% (range, 103–107%) at lactate concentrations of 6.29 and 11.89 mmol/l, respectively. No significant differences in recovery were observed between EDTA and lithium heparin anti-coagulated specimens ($p = 0.88$).

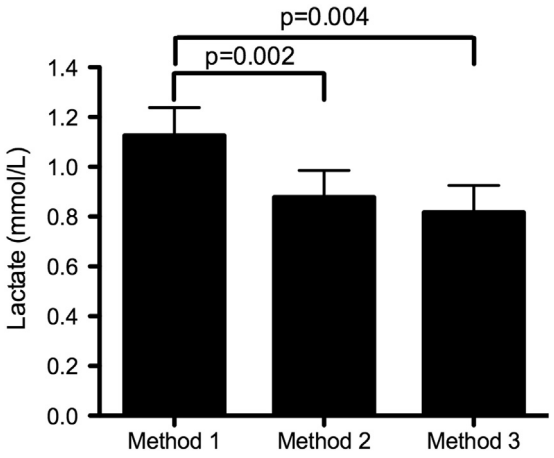

Fig. 1. Alternate methods of sample collection and processing using perchloric acid collection tubes significantly decrease lactate concentrations. Method 1 (reference): whole blood added to pre-chilled perchloric acid tube, incubated on ice for 10 min, and then centrifuged to obtain protein-free supernatant that was stored frozen for 24 h prior to testing. Method 2: whole blood added to room temperature perchloric acid tube then centrifuged to obtain protein-free supernatant that was stored frozen for 24 h prior to testing. Method 3: whole blood added to room temperature perchloric acid tube then stored frozen for 24 h and centrifuged immediately prior to testing to obtain protein-free supernatant. The error bars represent the standard error of the mean.

Precision was determined by measuring lactate in three replicates once each day for 10 days in each of 2 protein-free supernatants prepared by pooling residual patient samples. Within-laboratory imprecision was 6.1% and 1.1% at 1.58 and 10.89 mmol/l, respectively and between-day imprecision was 2.3% and 0.9% at the same respective concentrations.

The limit of blank (LOB) was determined using a sample without lactate, prepared by the addition of 1 ml of water to 2 ml of 8% perchloric acid. Lactate was measured ten times and the mean and SD were calculated as 0.002 and 0.006 mmol/l, respectively. The LOB was calculated as the mean added to three standard deviations, or 0.02 mmol/l.

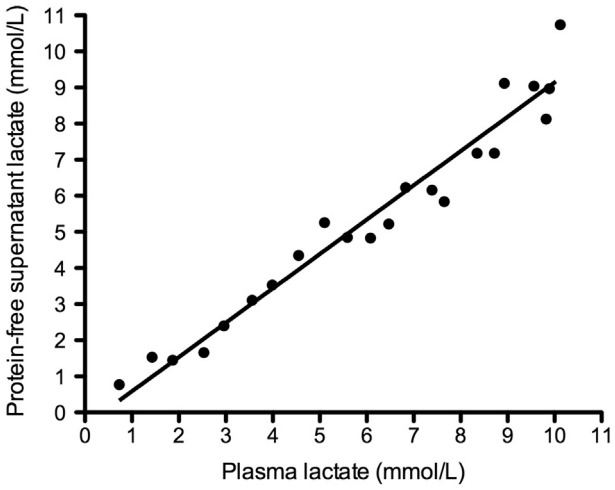

Fig. 2. Correlation plot of lactate measured in plasma vs. a protein-free supernatant. Deming regression analysis produced a slope of 0.95, a *y*-intercept of −0.37, and R^2 of 0.95.