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Continuous and simultaneous determination of venous blood metabolites

Anneli Andersson [a,1], Qun Chen [a,2], Leif Groop [b] , Leif Bülow [a] , Bin Xie [a*]

[a]Department of Pure and Applied Biochemistry, Lund University, P.O. Box 124, SE-22100 Lund, Sweden

[b]Department of Clinical Sciences, Diabetes and Endocrinology, Lund University, SE-20502 Malmö, Sweden

[*]Corresponding authors. Department of Pure and Applied Biochemistry, Lund University, Naturvetarvägen 14 , Box 124, SE-221 00 Lund, Sweden, Tel: +46 (0)46 2228261, Fax: +46 (0)46 2224611, E-mails: bin.xie@tbiokem.lth.se (B. Xie)

Abstract:

[1] Institute of Veterinary Biochemistry and Molecular Biology, University of Zürich, CH-8057 Zurich , Switzerland

[2] Institute of Endemic Diseases, Medical School of Xi'an Jiaotong University, 710061 Xi'an, China

Metabolic syndrome is associated with cardiovascular disease, type 2 diabetes mellitus (T2DM) and prediabetes. Metabolic syndrome is a cluster of interrelated clinical disorders. Difficulties in regulating glucose levels in blood are implicated in many of these disorders. Lactate, another energy metabolite, is produced under anaerobic conditions and can be used to monitor the balance between aerobic and anaerobic metabolism. Tested together, these metabolite levels can provide pro-diagnostic information that improves patient outcomes. Glucose and lactate were determined continuously and simultaneously in whole blood using a dual-channel thermal biosensor device in which one channel employed glucose oxidase for glucose analysis in comparison with lactate oxidase for lactate analysis in the others. No detectable clogging or interference was observed using venous blood samples. The linear detection range for both the glucose and lactate assays was 0.5-45 mM. The sampling rate of up to 24 samples per hour with assay cycle time of 2.5 min was achieved. Comparative analysis between our device and the HemoCue method showed an excellent correlation. The device was stable for hundreds of injections over a period of 45 days. The broad linear range, fast response and detection sensitivity are satisfactory for the clinical requirements, e.g. for diabetic or cardiovascular patients in intensive care units or surgical operation, where the tight control of blood glucose can decrease morbidity or mortality.

Graphical abstract

The dual-channel thermal biosensor for continuous and simultaneous determination of blood glucose and lactate.

Keywords: whole blood analysis, diabetes monitoring, continuous monitoring, thermal biosensor, glucose, lactate

Introduction

Metabolic syndrome is associated with cardiovascular disease, type 2 diabetes mellitus (T2DM) and prediabetes [1,2]. According to WHO 2016 Global report on diabetes, about 422 million adults were living with diabetes in 2014, and 1.5 million deaths were reported in 2012 [3]. Metabolic syndrome is a cluster of interrelated clinical disorders including obesity, insulin resistance, glucose intolerance, hyperglycemia and dyslipidemia. Prevalence increases with age and it is estimated to afflict 25% of the US population [4]. Metabolic syndrome is associated with imbalances in blood glucose levels which can be caused by insulin resistance, a physiological condition where insulin becomes less effective at lowering glucose levels resulting in hyperglycemia. When pancreatic β-cells are unable to produce sufficient amounts of insulin to counter act the effects of insulin resistance, blood sugar levels rise. This marks the progression towards full T2DM. Thus, one characteristic of diabetes is persistent or recurring hyperglycemia [5]. Hhyperglycemia has also been identified as a risk factor associated with increased trauma mortality rates and increased post-surgery infection rates [6,7].

Glucose is the primary metabolic energy source and blood levels are tightly regulated in order to maintain metabolic homeostasis. Not surprisingly, imbalances have catastrophic effects on the ability of the body to maintain homeostasis. In an intensive care environment, blood glucose levels can be controlled by injecting insulin. Glucose levels must be carefully monitored, since lowering the glucose level too much can result in a loss of consciousness and possibly insulin coma [8]. Patients undergoing diabetes therapy, as well as intensive care

patients have their glucose levels monitored continuously in order to eliminate this risk factor. Glucose levels are widely used as a measure of health status, but alone the results are often inadequate to make an accurate determination about the general health status of the individual. Analysis of additional metabolic biomarkers would provide a broader knowledge base which would reduce this uncertainty allowing for more accurate diagnosis.

One of the most relevant metabolic candidates is lactate. Lactate is produced primarily under anaerobic conditions. Anaerobic metabolism is far less efficient than aerobic metabolism, but it may produce energy much faster. Thus, lactate is a useful marker for determining how the body is regulating energy resources at any one moment. Increasing lactate concentrations is associated with stress. Lactate levels have been extensively studied in critically ill patients and it has been found to be useful in assessing the severity of illness or injury, as well as for monitoring the response to therapy [9]. Monitoring lactate levels has been shown to be predictive for identifying septic shock and multi-organ dysfunction [10]. These complications show up rapidly, typically within 24 h post-trauma, which emphasizes the need for continuously lactate monitoring during this critical period [11,12]. The simultaneous monitoring of blood glucose and lactate levels provides important information needed to assess the severity of an illness and for evaluating therapeutic efficacy [13].

Continuously simultaneous detection of glucose and lactate in blood has the potential to improve outcomes of patients. Simultaneous analysis has the additional advantage of simplifying sample handling and minimizing the number of blood samples needed to be withdrawn from the patient. Enzyme biosensors capable of simultaneously detecting two analytes are not commercially available. This is primarily due to interference between the enzymes used to detect glucose and lactate, mostly with the dehydrogenase. Even though the oxidase (e.g. glucose oxidase and lactate oxidase) has advantage in selectivity for the

substrate, the measurement ranges are normally limited by the amount of dissolved oxygen in the samples. Our group has developed a thermal biosensor, the Enzyme Thermistor (ET) with flow injected analysis. The device detects the heat generated by enzymatic reactions to quantitate the presence of specific analytes. The ET instrument has been used to detect a wide range of compounds from a variety of sources [14,15] and in a range of different experimental setups [17,18]. The ET has been previously used to detect glucose using very small blood samples with a miniaturized version ET [19-22]. Here, for first time we present the simultaneous determination of glucose and lactate in whole blood using a dual-channel ET device.

Experimental

Reagents and solutions

Glucose oxidase, (E.C.1.1.3.4) from Aspergillus niger (type X-S, 100-250 U mg^{-1} solid), catalase (EC 1.11.1.6) from bovine liver (2000-5000 U mg^{-1} solid) and lactate oxidase (E.C.1.13.12.4) from Pediococcus sp. (>20 U mg^{-1} solid) enzymes were purchased from Sigma-Aldrich. The D-(+)-glucose, L-lactic acid and bovine serum albumin (BSA, purified Fraction V) were also obtained from Sigma-Aldrich. Trisoperl, propylamino-derivatized controlled pore glass (CPG) beads with a particle diameter of 125-140 µm and a pore diameter of 59.55 nm were obtained from VitroBIO GmbH (Steinach, Germany).

The running buffer for the calibration curves was 0.1 M phosphate, pH 7.4 (PB). The whole blood measurements employed PBS (137 mM NaCl, 27 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4). Two additional reagents were included in the PBS buffer: 3.4 mM EDTA (to prevent coagulation), and 52 mM NaF (to halt any residual metabolic activity that might otherwise degrade the glucose and lactate). Venous blood samples from healthy

volunteers were drawn into 6 mL BD Vacutainer K2E tubes. EDTA and NaF were added to blood samples immediately after being drawn. Stock solutions of 80 and 100 mM L-lactate, D-glucose and mixed samples were prepared in PB. In order to minimize dilution effects, whole blood mock samples were prepared by adding 10 µL of concentrated substrate stock solutions to 200 µL of whole blood. All the solutions were preparing using de-ionized (18MΩ) water from a Milli-Q system (Millipore, Bedford, MA, USA).

Enzyme Column Preparation

To 500 mg of CPG, 10 mL of 2.5% glutaraldehyde in phosphate buffer was added and allowed to react 30 min under reduced pressure, follow by a 30 min incubation at normal pressure. The activated beads were washed and transferred to glass tubes containing the 2 ml of the enzyme in PB. The lactate measurement column contained catalase (13000 U g^{-1} CPG) and lactate oxidase (440 U g^{-1} CPG), and the glucose measurement column contained glucose oxidase (1000 U g^{-1} CPG) and catalase (13000 U g^{-1} CPG). The coupling reaction was allowed to proceed overnight at 4°C under mild agitation. The beads were decanted and 2 mL of PB containing 20 mg BSA was added to block any remaining sites. After one hour, the enzyme coupled CPG was washed and loaded into an ET column.

Instrumentation and assay conditions

The Enzyme Thermistor (ET) is a calorimetric flow injection analysis (FIA) based biosensor. This study uses the dual-channel ET model which employs a split flow scheme in order to determine two analytes simultaneously (Figure 1). The ET device consists of a peristaltic pump (Gilson Minipuls 2, Villiers-le-Bel, France) mounted with one tube for each channel, an injection valve (Valco Instrument Co., Texas, USA) and a dual-channel ET

instrument (Omik Bioscience AB, Sweden). In order to minimize heat leakage from the column, the inlet flow passes through a steel tubing that is wound around a copper tube (adiabatic shield) surrounding the column [23]. The thermistors are mounted on the exterior of the flow channels and are not in direct contact with the buffer or blood samples which reduce sample interference normally seen when whole blood samples are analyzed. After the introduction of blood into the system, the columns were washed thoroughly with the PBS buffer containing 10% ethanol to avoid bacterial growth and reducing the risk of clogging. The columns were then stored in PB at 4°C when not in use. The dual channel architecture makes it possible to simultaneously monitor the signal from each channel individually. A flow rate of 0.5 mL min^{-1} and a sample volume of 20 µL were used unless otherwise indicated. Triple measurements were performed for all the samples studied. The data was statistically analyzed for the standard deviation values and relative standard deviation (RSD) values.

Results

Assay Optimization

Our previous studies for determination of blood metabolites presented limited linear ranges [16,-19,20]. In an effort to improve the linear range, a series of experiments was performed to optimize sample volume and flow rate. Mixed samples of glucose and lactate were prepared in PB covering a concentration range from 80 mM down to 0.6 mM. Sample volumes of 20, 50 and 100 µL were investigated (Fig. 2). The optimal linear range was achieved using a 20 µL sample volume. We observed that reducing sample volume resulted in an increase of the sample dispersion and dilution during the flow course, that caused the extension of the measurement range [16]. The effect of flow rate was also investigated. Three flow rates were examined: 0.25, 0.5 and 0.6 mL min^{-1} for each assay. The results indicated

that flow rate in this range insignificantly affect the linear range. Therefore 0.5 mL min^{-1} was chosen in these studies.

Determination of glucose and lactate in buffer and whole blood

A calibration curve using whole blood samples spiked with substrate was prepared and analyzed (Fig. 3). The linear ranges for both glucose and lactate were 0.5-45 mM. The sampling rate of up to 24 samples per hour was achieved corresponding to an assay cycle time of 2.5 min.

The extended linear range and reduced response in blood measurements were observed as compared with the same concentration of the samples in buffer where the linear range was smaller and the response was higher than that with the blood samples (see figure 2). The recovery of 87% and 73% for glucose and lactate, respectively, were obtained when comparing the sensitivity under the same conditions. This was most probably due to the matrix effect in whole blood component determinations, such as hematocrit, in comparison with conditions when using pure buffer analysis [24].

No cross-talk was seen between the glucose and lactate reactions using an equal split-flow injection and two independent sensing devices (dual-channel ET). However, effect of pH on the response was observed for blood samples within the clinically relevant blood pH levels (Fig. 4). Possible interferences of commonly occurring substances in blood were analyzed in this study. Insignificant effects were observed for glucose and lactate measurements using whole blood mock samples spiked with 0.5 and 2.5 nM insulin, 1 mM ascorbic acid, 10 mM pyruvate, and 10 mM urea, respectively.

The lactate oxidase response was smaller than that for glucose oxidase in whole blood samples. This was due to the fact that the whole blood generally contained higher concentration of glucose (about 5 mM) than that of lactate (about 2 mM) for healthy people. In addition, NaF, which was added to the blood samples, proved to slightly inhibit lactate oxidase [9,19].

Reproducibility and stability

Continuous monitoring of patients in intensive care or surgical operation requires an accurate and reliable measurement system that could function for several hours with frequent sampling and determination without need of any recalibration. In order to verify that our system could meet these demands, the reproducibility and stability of glucose and lactate measurements were carried out both in buffer and blood samples. Ninety samples in PBS solution containing 10 mM glucose and 5 mM lactate were injected with sampling rate of 12 injections/hour (Fig 5A). The relative standard deviation (RSD) of the glucose and lactate measurements was 3.84% and 5.71%, respectively. These results are acceptable in comparison with our previous results using a mini-ET in which a better RSD was obtained for a single analyte determination [19]. The same study was performed with blood samples. The native concentrations of glucose and lactate in the blood sample were first determined using the ET, and then spiked with glucose and lactate to a final concentration to approximately 10 mM and 5 mM, respectively. For this experiment, 100 injections were carried out (Fig. 5B). The RSD values were 10.5% and 12.1% for glucose and lactate, respectively.

The long-term stability and biocompatibility of the system were investigated as well. The response over time was registered for 45 days using the same stock solutions diluted into

a final concentration of 5 mM glucose and 5 mM lactate. During this period, the columns were used regularly for measurements of samples in buffer and blood. The response changes of +4.82% and -6.85% for glucose and lactate were obtained, respectively.

Reference method

In order to determine how the ET measurements, relate to commercial devices, a comparative study was carried out. Self-monitoring of blood glucose is routinely done by diabetic patients using the finger pricking method [25]. The HemoCue Glucose 201 RT (HC) glucose measurement device was chosen for these studies. The HC instrument has an operational glucose concentration range of 0-22.2 mM and a sample volume size of 5 µL [13]. Based upon 18 measurements of blood samples in the range of 3-26 mM glucose, the correlation coefficient between the two methods was 0.98, and the linear equation was ET=HC-1.24 (Fig. 6).

There was a slightly lower value of the ET measurements as compared to the HC. The mean bias was -1.29 mM with -3.54 to 0.96 mM as the limit of agreement between the ET and HC. The proper explanation for the negative bias observed with the ET could be due to the dispersion of blood samples that resulted in a slight sample dilution during the flow course [19].

Discussion

Previous studies in our lab showed that sample volume and flow rate affected the linear range and sensitivity of the ET assay [19,20]. The present study further verified and improved the analytical performance of the ET biosensor. In this study, we chose to use the

HC instrument as reference method for glucose analysis. In the case of lactate, we have previously demonstrated that the ET measurements show excellent correlation with the reference method LACT MPR 1 [19] in whole blood and no further follow up was therefore made here. The flow injection based enzyme biosensors are dependent upon balance between catalytic efficiency of the enzymes and analytical efficiency of the flow injected system, such as flow rate, sample volume, response time.

The choice of using the oxidases for glucose and lactate analysis in this study is due to the fact that these enzymes are more specific and selective on the substrate than the corresponding dehydrogenases. This is particularly important in whole blood analysis where multiple substrates could generate interferences frequently encountered using the dehydrogenase based measurements. However, the oxygen dependent oxidase reactions normally have a limited linear range of analysis. In this study, the GOD and LOD reactions shared the dissolved oxygen in the split flow injection process, but the availability of this gaseous cosubstrate was sufficient for the simultaneous determination of glucose and lactate.

Our results have demonstrated the feasibility for the clinical monitoring of patient with sufficient linear ranges (0.5-45 mM) for both glucose and lactate and sampling rate of up to 24 samples per hour with assay cycle time of 2.5 min. This was due to the optimally balanced conditions for the response time, measurement range and resolution using a small sample volume of 20 μL that made increased dispersion/dilution during the flow course of the samples (Fig. 2). In addition, modification of the dual-channel ET device has improved efficiency for the effective oxygen utilization and sample mixing during the reactions, so that to prevent the molar concentration of the substrate from exceeding the concentration of the dissolved oxygen in the sample [16]. In addition, the optimal flow rate, as another factor, was selected in comparison between three flow rates: 0.25, 0.5 and 0.6 mL min^{-1}. Although the

flow rate had little effect on linear range in this case, but affected determination response when considering clinical applications in which assay cycle time of 2.5 min could be achieved with our solution.

The dual-channel ET was superior to other enzyme biosensors for the simultaneous detection of glucose and lactate in whole blood samples, because the thermal biosensor employed a non-contact model between the sensor (thermistor) and flow sample (blood), that avoided direct interferences from the co-substances in blood and eliminated the need for routine recalibration which is otherwise required as compared with electrochemical and optical sensors where chromophores or electroactive species often interfere with the measurements. The enzymes (GOD and LOD) used in this study were very specific and no significant cross-talk between the two reactions was observed. The enzyme columns could efficiently function for over 45 days, as compared to the standard YSI dual analyzer where the enzyme membranes have to be exchanged every 21 days [26].

The use of spherical CPG with size over 100 μm in diameter provides two advantages, high surface area for immobilization and porous flow-path for blood cells which might otherwise results in clogging. One aspect that remains problematic is injection valve clogging. This needs to be improved, for instance by adding heparin in the solution in order to increase the precision and reliability of the measurement. The linear range and sampling rate obtained for both lactate and glucose were sufficient for clinical applications. The repeatability for whole blood measurements needs to be improved in order to meet the demands for a long-term continuous monitoring in a clinical environment.

To conclude, in this study we have verified that the same operational conditions can be used for both oxidases allowing simultaneous determination of glucose and lactate. It has also been demonstrated that enough dissolved oxygen is available for two independent

enzymatic reactions. In an extension, this can act as a platform for more extensive monitoring of multiple analytes in whole blood using oxidases as enzymatic system.

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Fig. 1 Schematic of the dual channel thermal biosensor.

Fig. 2 Flow rate and sample size calibration curves for glucose (A) and lactate (B).

Fig. 3 Calibration curves for glucose and lactate in whole blood.

Fig. 4 Calibration curves for glucose and lactate between pH 6-8.

Fig. 5 Repeatability of glucose and lactate assays in buffer (A) and whole blood (B).

Fig. 6 Comparison between the thermal biosensor and HemoCue for whole blood glucose analysis.

Highlights

1. Operational conditions have been identified for two separate oxidase reactions which share the dissolved oxygen in a dual-channel system. Adequate linear ranges for both glucose and lactate have been achieved.

2. This was the first work for the simultaneous determination of two blood metabolites with insignificant crosstalk.

3. Continuous and long term operation with good stability and reproducibility were demonstrated.

4. Continuously frequent determination of blood more than 24 sample/hour (or assay cycle time of 2.5 min) could be performed.

5. Insignificantly direct interferences from blood co-substance, so that to be able to last for a long stable operation for over 100 blood measurements with clinically acceptable RSD for glucose (3.84%) and lactate (5.71).