Urea and Lactate Determined in 1-µL Whole-Blood Samples with a Miniaturized Thermal Biosensor

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A miniaturized flow-injected thermal biosensor was developed for the determination of urea and L-lactate in undiluted blood in 1-µL samples. The sensor employed a small enzyme column constructed of stainless steel tubing and microbead thermistors. Urease and lactate oxidase/catalase were separately immobilized onto controlled-pore glass beads, which, in turn, were charged into the enzyme column. With a flow rate of 70 µL/min, linear analytical ranges from 0.2 to at least 50 mmol/L and 0.2 to 14 mmol/L were obtained for urea and lactate, respectively. The relative standard deviations (CVs) for measurements of analyte in buffer were 0.91% for urea and 1.84% for lactate. For urea in whole blood, the CV for 50 determinations was 4.1%. Contrived samples containing various concentrations of urea and L-lactate in whole blood were determined with this sensor and with a spectrophotometric method. Comparisons of the results gave correlation coefficients of 0.989 and 0.984 for 30 blood urea and 30 blood lactate assays in concentrations ranging from 4 to 20.9 mmol/L and from 1.7 to 12.7 mmol/L, respectively.

Indexing Terms: immobilized enzymes/flow-injection analysis

The determination of such metabolites as glucose, lactate, and urea in whole blood is of central importance in clinical diagnostics. This information provides guidelines for determining our general state of health. Although routine monitoring of personal health allows the detection of disease states at an early stage, such monitoring is expensive and inconvenient. Development of a simple, inexpensive, and reliable analyzer for use in decentralized health facilities is of special interest. Small sample volumes and small size would be preferred for this type of instrument. A great deal of effort has been devoted to the development of such devices, especially for glucose determinations (1–10).

Biosensors have many advantages, including convenient operation, rapid response, and low cost. The majority of these systems are based on electrochemical or optical detections (11–17), which provide good sensitivity and selectivity. However, their application in whole-blood measurements is limited because of interference from electroactive species, ions, turbidity, and products. Moreover, transducers, such as electrodes, require frequent recalibration for long-term operation.

As an alternative, we have designed and constructed a miniaturized flow-injected thermal biosensor based on the conventional enzyme thermistor (18, 19). Compared with other biosensors, thermal biosensors are broadly applicable and are intrinsically insensitive to the optical and electrochemical properties of the sample. Moreover, because the thermal transducers (e.g., thermistors) are usually insulated from the fluid and intrinsically are very stable, these biosensors do not require frequent recalibration during long-term operation. The feasibility of using such a system for determining whole-blood glucose has previously been demonstrated with a micro-column-based thermal biosensor (20). By reducing the sample volume, glucose concentrations ≤20 mmol/L in whole blood could be analyzed without any pretreatment of the sample. This extended linear range was attributed to a relative increase in sample dispersion (21).

Here, we report the development of a method for the determination of urea and L-lactate in undiluted whole-blood samples by use of a similar miniaturized thermal biosensor. As in our previous blood glucose studies, we used 1-µL sample volumes to extend the linear range of the analytical range into the relevant physiological concentration range.

Materials and Methods

Materials

L-Lactate oxidase (EC 1.1.3.2; 39 kU/g) from Pediococcus sp., urease (EC 3.5.1.5; 66 kU/g) from jack beans, and catalase (EC 1.11.1.6; 19 000 kU/g) from beef liver were purchased from Sigma Chemical Co. (St. Louis, MO). Human blood samples were taken from healthy volunteers. Analytical grade L-lactate (sodium salt; Sigma) and urea (E. Merck, Darmstadt, Germany) were used as the standards; all other reagents were analytical grade. The test kits for the comparative measurements of blood urea (Urea Granulitest 15 Plus; Diagnostica Merck, Darmstadt, Germany) and lactate (LACT MPR 1; Boehringer Mannheim, Mannheim, Germany) were supplied by the manufacturers. Propylamino-derivatized controlled-pore glass beads (CPG; particle diameter 125–140 µm, pore diameter 50 nm) was obtained from Steinachglas, Steinach, Germany. The micro-bead thermistors (K19/20%/12K) used as the temperature transducer were purchased from Siemens, München, Germany.

Preparation of the Enzyme Matrixes

All enzymes used in the study were covalently immobilized on spherical CPG beads as follows: The propylamino-CPG was activated with 25 mL/L glutaraldehyde in 0.1 mol/L sodium phosphate buffer, pH 7.0, at room temperature for 1 h under reduced pressure, washed

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exhaustively on a Büchner funnel with distilled water, and finally stored in water at 4°C. Lactate oxidase (~50 U) and catalase (>20 000 U) were added to 50 mg of wet, activated CPG suspended in 500 μL of buffer. The coupling was allowed to proceed at 4°C overnight on a shaker. Before packing it into a column, we thoroughly washed the enzyme preparation with phosphate buffer, then packed ~25 μL of the matrix into each column. Similar procedures were used for urease immobilization, adding 800 U of urease to 0.5 g of wet, activated CPG suspended in 0.5 mL of buffer.

Preparation of Samples

The L-lactate samples were dissolved in a buffer containing 137 mmol/L NaCl, 2 mmol/L EDTA, and 100 mmol/L sodium phosphate (pH 7.0). Urea samples were prepared in the same buffer but also containing 2 mmol/L β-mercaptoethanol. Urea and lactate calibrators were prepared in their respective buffers. Venous blood samples were collected into EDTA-containing tubes and stored on ice until the measurements were performed.

Biosensors

The sensor device, 24 mm (o.d.) × 54 mm, was composed of three parts: a cylinder, and two cover mounts that could be easily disassembled for convenient access to the enzyme column (Fig. 1). The enzyme column, 15 mm × 1.5 mm (i.d.)/1.7 mm (o.d.) was constructed of stainless-steel tubing and placed in the middle of the cylinder. A thin copper foil (0.1 mm thick), onto which a stainless-steel tubing [0.3 mm (o.d.)] was tightly and densely coiled, surrounded the column. This foil served as an adiabatic shield by preserving the heat of the outlet stream. The adiabatic layer was insulated by a 2-mm-thick layer of Plexiglas, which in turn was covered with an aluminum jacket, which functioned as a heat sink. Stainless-steel tubing [0.4 mm (o.d.)] was wound in parallel coils around the inside surface of the jacket to equilibrate the temperature of the incoming fluid. The microbead thermostats were mounted onto gold capillary tubings [0.3 mm (o.d.)] with heat-conducting epoxy. These gold tubings together with the thermosts functioned as temperature-sensitive elements and were positioned close to the enzyme column. The entire aluminum cylinder and the two cover mounts were then enclosed by a 3-mm-thick layer of Plexiglas.

Thermometric Assay

The setup for the thermometric assay consisted of a peristaltic pump (Altelea pump unit C-4V; Ventur Teknik AB, Ultran, Sweden), an HPLC valve suitable for 1-μL samples (Type C14W; VIGI AG, Valco Europe, Schenkon, Switzerland), a preheat sink, the sensor device, a dc-coupled Wheatstone bridge equipped with a chopper-stabilized amplifier (constructed at our institute), and a chart recorder. During the operation, the sensor was kept in an aluminum box insulated with polyurethane foam to minimize interferences from changes in the environmental temperature. The effect on the thermal signal detection was insignificant for a change of about ±2°C at room temperature (normally 22°C), even without temperature control. Because the sample volume was only 1 μL in an assay run at a flow rate of 70 μL/min, there was good margin for temperature equilibration before the sample reached the enzyme column via the heat-sink inside the aluminum box. The temperature change corresponding to the enzyme reaction (usually in the range of 10⁻²–10⁻¹°C) taking place in the column was registered with the Wheatstone bridge. At maximum sensitivity, the signal changes 100 mV per 10⁻³°C.

Comparison Assays

For comparison, we also used methods based on ultraviolet spectrophotometric detection to determine the urea and L-lactate concentrations in whole blood.

With the Urea Granutest 15 Plus test kit, the plasma required for the assay was prepared by centrifuging the blood specimen for ~5 min at 1000g. The determination is based on the reactions catalyzed by urease and glutamate dehydrogenase (EC 1.4.1.3), with the accompanying consumption of NADH being detected spectrophotometrically at 340 nm (22). These determinations were assumed to correspond to the concentration of urea in whole blood, there being only slight differences in urea concentration between plasma and whole blood (23). In the LACT MPR 1 test kit, lactate plus NAD⁺ were converted to NADH and pyruvate by lactate dehydrogenase (EC 1.1.1.27); the pyruvate in turn was broken down by alanine aminotransferase (EC 2.6.1.2). The NADH produced was measured at 340 nm (24). Before the assay the sample was deproteinized by mixing 1 mol/L perchloric acid with an equal volume of blood and centrifuging; the supernate was used for the determination. The results obtained with this method correspond to those for whole blood, according to the manufacturer’s calibration procedure.

Principles

According to the urease reaction (25), urea is hydrolyzed into the products below by urease in neutral phosphate buffer:

![Fig. 1. Schematic diagram of the sensor construction.](image)
\[3\text{H}_2\text{O} + (\text{NH}_2)_2\text{CO} \xrightarrow{\text{Urease}} 2\text{NH}_4^+ + \text{HCO}_3^- + \text{OH}^- + \Delta H\]

where \(\Delta H\) is the enthalpy change of the reaction.

For the lactate assay, lactate oxidase and catalase are used to convert L-lactate:

\[\text{L-Lactate} + \text{O}_2 \xrightarrow{\text{Lactate oxidase}} \text{pyruvate} + \text{H}_2\text{O}_2 + \Delta H_1\]

\[
\downarrow \text{Catalase}
\]

\[\text{H}_2\text{O} + \frac{1}{2}\text{O}_2 + \Delta H_2\]

where \(\Delta H_1\) and \(\Delta H_2\) are the enthalpy changes produced by the lactate oxidase and catalase reactions, respectively. This reaction scheme provides additional heat generated by the reduction of \(\text{H}_2\text{O}_2\) (100 kJ per 1 mol/L) and by the partial regeneration of \(\text{O}_2\). This extends the linear range of the lactate assay and eliminates the presence of \(\text{H}_2\text{O}_2\), which could otherwise affect the stability of the immobilized enzymes (26).

**Results and Discussion**

**Properties of the Sensor**

The linear range and the operational stability of the sensor were evaluated with lactate and urea calibrators in buffer. As mentioned above, the hydrolysis of urea is limited only by the amount of enzyme in the column. In contrast, the lactate assay depends also on the amount of dissolved oxygen. In this study, we used the 1-\(\mu\)L samples to extend the linear range of the lactate analysis.

The thermal calibration curves for the urea and L-lactate calibrators were used to evaluate the results from whole-blood measurements over the ranges of 0.5–50 mmol/L for urea and 0.5–20 mmol/L for L-lactate. For urea a broad linear range at least 50 mmol/L was obtained (Fig. 2, top). As the results indicate, the enzyme columns had adequate catalytic capacity for full conversion of the corresponding substrates even though the amount of enzymes (26 \(\mu\)L column volume) used was much less than that (>200 \(\mu\)L) in the conventional enzyme thermistors. The linear range of L-lactate analyses extended only to 14 mmol/L (Fig. 2, bottom), limited by the amount of dissolved oxygen available. Nevertheless, this concentration range is adequate to measure lactate concentrations typically found in the blood, the normal physiological range being 0.55–2.22 mmol/L (5–20 mg/100 mL) for lactate (for urea, it is 1.33–4.33 mmol/L (8–26 mg/100 mL)) (27).

During exercise, however, the concentration of lactate in blood often exceeds 14 mmol/L (28). In similar analytical systems, samples with extremely high concentrations of analyte can be assayed by further reducing the sample volume (20, 21) or by sample dilution. In addition, we found that the linear range and the sensitivity of lactate assay were significantly affected by the amount of immobilized catalase and lactate oxidase. Increasing the amounts of immobilized lactate oxidase increased the peak height and decreased the linear range, an indication that the oxygen diffusion rate in the buffer limited the reaction rate. On the other hand, decreasing the amount of immobilized catalase resulted in a decrease in both the linear range and the peak height. This insufficient conversion of the hydrogen peroxide by catalase in turn reduced the oxygen supply and decreased the total thermal output.

The lower detection limit (signal-to-noise ratio \(\geq 2\)) was 0.1 mmol/L for both analytes in 1-\(\mu\)L samples. Increasing the sample volume lowered the detection limit but only at the cost of decreasing the linear range.

The linearity and sensitivity of flow-injected biosensors are affected by the flow rate. This is particularly true for the extensively miniaturized thermal biosensors since the enzymatic catalysis and the thermal detection are time dependent. Too high a flow rate reduces the sample residence time, which in turn reduces the substrate conversion efficiency; consequently, the thermal response is decreased. On the other hand, reducing the flow rate resulted in poor linearity because of increased heat leakage (21). In this system, we found the
optimal flow rate to be \( \sim 70 \) \( \mu \text{L/min} \); this allowed a sampling rate of 40 samples per hour.

The operational stability of the biosensor was evaluated by performing > 100 manual injections of calibrator solutions (50 mmol/L urea and 10 mmol/L L-lactate) for each enzyme column. The relative standard deviations (CVs) were 0.91% for the urea assay and 1.84% for the L-lactate assay. Comparison of the measurement series showed that, at the same concentration, the L-lactate response is about three times that of urea. This result is consistent with other studies (18, 29) in reporting enthalpies of \( \sim 180 \) kJ per mol/L for lactate catalyzed by lactate oxidase/catalase and 61 kJ per mol/L for urea catalyzed by urease in phosphate buffer.

Factors Affecting the Assay

The determination of urea and lactate are affected by the enzyme activity and the transducer stability. Because the thermists were very stable and isolated from the buffer solution, the most important factors affecting the determinations were those that influenced the enzyme reaction, such as enzyme inhibitors, pH changes, buffer composition, and nonspecific heat.

The most common inhibitors of urease are heavy metal ions, and some metals can inactivate the enzyme at very low concentrations. The effect could not be avoided in this flow system, given the use of metal tubing in the heat sink and the inlet connections; moreover, whole blood itself contains free metal ions. Therefore, we included EDTA to reduce the inhibitory effects of the metal ions. Oxidants can also affect urease activity, particularly in long-term operation. As we found previously (30), the antioxidant \( \beta \)-mercaptoethanol stabilizes urease. Similar results were found in this study with use of phosphate buffer containing 2 mmol/L EDTA, 2 mmol/L \( \beta \)-mercaptoethanol, and 137 mmol/L NaCl (not shown). We also investigated the effect of other compounds — e.g., HCO\(_3\) (10–50 mmol/L), NaCl, and KCl — on the urease reaction. Including HCO\(_3\) did not affect the thermal response, but NaCl (\( > 50 \) mmol/L) and KCl (\( > 5 \) mmol/L) did. These ion effects are discussed below.

The effect of pH on the lactate oxidase reaction was studied with two different buffer systems, phosphate buffer (pH 6–8) and citrate buffer (pH 5–6). In the experiment, the same buffer was used as running eluent and to prepare the L-lactate calibrators (5–20 mmol/L). The calibration curves obtained with these two buffer systems showed no significant differences between them. Furthermore, no significant interference was observed from HCO\(_3\) (2–100 mmol/L) or other metabolites, such as L-ascorbic acid (1–6.8 mmol/L), pyruvate (0.05–0.7 mmol/L), and oxalate (0.1–1.5 mmol/L), in phosphate buffer, pH 7.0. However, the peak height increased because of the heat of solvation when lactate calibrators containing KCl or NaCl were injected. To ascertain the relative contribution from these salts on the thermal signal, we analyzed several samples containing various amounts of NaCl (10–500 mmol/L) and KCl (10–500 mmol/L) in citrate (pH 6.0) and phosphate (pH 7.0) buffers. Fig. 3 shows the different responses. As can be seen, this nonspecific heat was related to the concentration of the salts. The thermal response was affected more by the citrate buffer than by the phosphate buffer at equivalent salt concentrations, and the effect was more pronounced with KCl than with NaCl. The relative effect of these salts decreased as the sample concentration increased. In whole-blood measurements, the low concentration of KCl (\( \sim 3.7 \) mmol/L) had an insignificant effect. However, given the considerably higher concentration of NaCl (\( \sim 137 \) mmol/L) in whole blood, it was necessary to supplement the buffer with NaCl to eliminate nonspecific heat effects for injection of whole-blood samples.

Assay of Blood Samples

Human venous whole-blood samples were stored on ice after collection to stabilize the concentration of the analytes. This is particularly important for lactate determinations, because its concentration increases dramatically after being withdrawn from the body. For the comparison studies, we divided each blood sample into two portions and immediately analyzed them with the biosensor and the spectrophotometric method.

Urea in whole blood was determined in 0.1 mol/L phosphate buffer (pH 7.0) containing 2 mmol/L EDTA, 2 mmol/L \( \beta \)-mercaptoethanol, and 137 mmol/L sodium chloride. Blood samples containing various concentrations of urea were prepared by adding 1–15 \( \mu \)L of a 200 mmol/L urea calibrator to 200-\( \mu \)L aliquots from a single whole-blood specimen. The urea concentration in whole blood was determined by comparing the thermal response from these blood samples with the calibration curve established from urea buffer calibrators. We then compared these results with the blood urea sample determinations obtained with the spectrophotometric method (Fig. 4). The correlation between the two methods was \( r = 0.989 \) for 30 blood samples ranging in concentration from 4 to 20.9 mmol/L urea. The standard

![Fig. 3. Contribution of KCl and NaCl to nonspecific thermal responses of the lactate sensor in citrate (pH 6) and phosphate buffers (pH 7), respectively.](CLINICAL CHEMISTRY, Vol. 40, No. 12, 1994 2285)
deviations for the slope ($S_b$) and the intercept ($S_a$) were 0.0267 and 0.3581, respectively. The use of the appropriate $t$-value for 28 degrees of freedom ($t = 2.04$) gave the 95% confidence limits for the intercept and slope as $a = 0.4036 \pm 0.7305$ and $b = 0.96324 \pm 0.05448$. The CV was 4.1% for 50 determinations made on the same day of one blood sample containing a mean value of 0.37 mmol/L urea. We used the same column for blood urea determinations for about a week at room temperature without significant decrease in the thermal response. The analysis of additional samples should be possible, given that no clogging in the column was observed after 50 whole-blood sample injections.

The determination of whole-blood lactate was carried out similarly. The buffer used in these analyses consisted of 2 mmol/L EDTA and 137 mmol/L NaCl in 0.1 mol/L phosphate buffer, pH 7.0. Blood-based lactate calibrators were prepared as described for the urea calibrators. The results obtained from these blood sample measurements were compared with the calibration curve for the lactate calibrators in buffer and with the results of the comparison method. A correlation coefficient of 0.984 was obtained between these two methods for 30 lactate assays ranging in concentration from 1.7 to 12.7 mmol/L (Fig. 4). In this study, the standard deviations for the slope ($S_b$) and the intercept ($S_a$) were 0.0320 and 0.2528, respectively. The 95% confidence limits for the intercept and slope were: $a = 0.0881 \pm 0.5157$ and $b = 0.92827 \pm 0.06528$. No systematic errors were observed in the comparison study, although others have reported a gradual, time-dependent increase in the lactate concentration in blood samples (31). This drift in lactate concentration in blood samples makes the performance of reproducibility studies difficult to interpret. However, we find it worth noting that our method correlates well with the LACT MPR 1 test kit.

In conclusion, we have demonstrated the feasibility of developing a universal biosensor for determining whole-blood metabolites by measuring blood urea and lactate in a miniaturized flow-injected thermal biosensor. The use of small sample volumes and a spherical, rigid enzyme support material with uniform particle size allowed virtually all of the blood cells to pass through the enzyme column. No clogging was observed for the number of samples (up to 100) studied here, irrespective of hematocrit. The device requires only occasional calibration, and the universality of the method makes it simple to implement. The simple reaction scheme in this system does not require any special reagents or chromophores that can cause interference. Therefore, the method shows promise for determining many other blood metabolites besides glucose, urea, and lactate. The linear range of the oxidase reactions, exemplified by the lactate oxidase reaction described here, was extended by using very low sample volumes. Alternatively, it might be possible to extend the linear range of the oxidase reaction by electrochemically regenerating an electron mediator, such as ferrocene, to replace oxygen in the buffer (32).

The approach described here could easily be applied to the simultaneous analysis of multiple analytes (30). The use of immobilized enzyme columns provides an economical alternative to single test kits because each column can be used for at least 100 determinations. In the future, reliable, inexpensive, and convenient portable devices based on this principle could be developed for decentralized and home health monitoring.

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