Kinetic Enzymic Assay for \( \delta(\neg) \)-Lactate, with Use of a Centrifugal Analyzer

Carl W. Ludvigsen,1,3,6 Joseph R. Thurn,5 Gordon L. Pierpont,2,4 and John H. Eckfeldt1,3

The \( \delta(\neg) \)-isomer of lactic acid appears to cause a form of metabolic encephalopathy experienced by patients who have had jejunoileal bypass for morbid obesity. However, analysis for \( \delta(\neg) \)-lactate is not routinely available in clinical or reference laboratories. We describe an enzymic centrifugal-analyzer assay for \( \delta(\neg) \)-lactate in plasma or serum, with use of \( \delta(\neg) \)-lactate dehydrogenase. The method involves two-point kinetic calibration and preincubation of specimen and NAD+, thus eliminating the need for specimen-blanking or protein-precipitating pretreatment. This rapid, accurate, and precise assay should be helpful in evaluating patients with "short-bowel syndrome" who display confusion, lethargy, ataxia, or other central nervous-system disturbances that may be ascribable to \( \delta(\neg) \)-lactic acidosis.

Additional Keyphrases: metabolic acidosis • anion gap • monitoring therapy • ketoacidosis • jejunoileal bypass • bacterial overgrowth • short-bowel syndrome • nervous-system disorders

Because the C2 carbon of lactic acid is chiral (optically active), there exist two enantiomers of lactic acid, which rotate plane-polarized light in equal but opposite directions. Most of the common clinical "lactic acidoses" result from in vivo production of \( \Delta(+) \)-lactate (S absolute configuration), caused by a variety of pathophysiological processes. Thus, in most of the commonly used clinical laboratory methods, \( \Delta(+) \)-lactate dehydrogenase (EC 1.1.1.27) is used in this assay, and only \( \Delta(+) \)-lactate is measured. \( \delta(\neg) \)-Lactic acid (R absolute configuration) has not been considered to be of significant clinical importance in humans until 1979, when a patient was reported (1) who had had most of his jejunum removed because of superior mesenteric artery thrombosis with small bowel gangrene and about two years later had presented with repeated episodes of fluctuating encephalopathy and metabolic acidosis. \( \delta(\neg) \)-Lactic acid was eventually discovered to be the cause of the patient's symptoms when his urine was examined for an unusual organic acid, thought possibly to come from colonic bacteria. This acid had a \( \mathrm{pK}_a \) and gas-liquid chromatographic properties identical to those for \( \Delta(+) \)-lactic acid. The usual gas-liquid chromatography involving derivitization with non-chiral compounds will measure \( \Delta(+) \) and \( \delta(\neg) \)-lactic acid as identical compounds. The enanitomers of lactic acid may be separated by gas-liquid chromatography by use of a chiral stationary phase or by use of derivitization of the enaniomers into diastereomers with a chiral alcohol, usually \( l(-) \)-menthol (2). An easier approach for determination of \( \delta(\neg) \)-lactic acid is to use a specific \( \delta(\neg) \)-lactate dehydrogenase (EC 1.1.1.28) from microorganisms (3–5).

Subsequently, \( \delta(\neg) \)-lactic acidosis has been shown to be the agent causing a fluctuating encephalopathy characterized by dizziness, ataxia, headache, lethargy, memory lapses, and metabolic acidosis in several patients who have had jejunoileal bypass for morbid obesity (6–9). Results of routine clinical laboratory enzymic determinations of "lactic acid," measuring only \( \Delta(+) \)-lactic acid, were normal in these patients. In ruminants, which can get \( \delta(\neg) \)-lactic acidosis from over-feeding with grain (10), and in man, the degree of encephalopathy appears to correlate with the concentration of \( \delta(\neg) \)-lactate in serum. The \( \delta(\neg) \)-lactate is a metabolic product of many bacteria, especially Gram-positive, non-spore-forming anaerobic bacteria (7). It is neither produced nor metabolized by mammals to any great extent (see Discussion). In man, the episodes seem to be recurrent. Though treatment with oral non-absorbable antibiotics reverses the symptomatology concomitant with a decrease in the concentration of \( \delta(\neg) \)-lactate in serum, the episodes often subside spontaneously, probably owing to voluntary restriction of oral food intake (1).

The assay we describe here allows almost any clinical laboratory to rapidly identify \( \delta(\neg) \)-lactate as the cause of this encephalopathy, and should avoid long and expensive search for other etiologies of these patients' symptoms.

Materials and Methods

Materials

\( \delta(\neg) \)-Lactic acid (stock no. 826-10), lithium \( \delta(\neg) \)-lactate (no. L1000), 10-mg pre-weighed vials of NAD6 (no. 280-110), \( \delta(\neg) \)-lactate dehydrogenase from Lactobacillus leichmannii (no. L3504), and 0.6 mol/L glycine–0.5 mol/L hydrazine buffer, pH 9.2 (no. 826-3), were obtained from Sigma Chemical Co., St. Louis, MO 63178. Ultracentrifugation was performed at 140,000 \( \times \) g for 10 min, in an Airfuge (Beckman Instruments, Palo Alto, CA 94304). We used a Multistat III microcentrifugal analyzer (Instrumentation Laboratory, Lexington, MA 02173) with the following reagents and procedure:

**NAD6 reagent.** Combine 1 mL of Sigma glycine–hydrazine buffer and 1 mL of water with 10 mg of NAD6. This mixture is stable for at least 48 h at 4 °C.

**\( \delta(\neg) \)-Lactate dehydrogenase reagent.** Combine 100 units of \( \delta(\neg) \)-lactate dehydrogenase suspension (approximately 100 \( \mu \)L), 1 mL of Sigma glycine–hydrazine buffer, and 1 mL of water. This mixture is stable for at least 48 h at 4 °C.

**Working \( \delta(\neg) \)-lactate standard, 10 mmol/L.** First prepare an approximately 100 mmol/L stock standard from \( \delta(\neg) \)-lactic acid crystals. Because of the hygroscopic nature of these crystals, combine 1 mL of this stock standard with 4 mL of water and titrate with 100 mmol/L sodium hydroxide, with use of a pH meter, to assign an accurate \( \delta(\neg) \)-lactic acid concentration. Prepare the working \( \delta(\neg) \)-lactate standard by dilution with water. Alternatively, prepare a working \( \delta(\neg) \)-lactate standard directly from lithium \( \delta(\neg) \)-lactate. Store frozen at -70 °C, at which temperature it is stable for at least six months.

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From the Laboratory1 and Medical3, Services, Veterans Administration Medical Center, Minneapolis, MN; and Departments of Laboratory Medicine and Pathology,4 Medicine,4 and Medical School,8 University of Minnesota, Minneapolis, MN.

6 Current address: Dept. of Pathology & Laboratory Medicine, University of Nebraska Medical Center, Omaha, NE 68105.

Direct correspondence to Dr. Eckfeldt at Laboratory Service (112), Veterans Administration Medical Center, Minneapolis, MN 55417.

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Controls. Prepare a low serum control from pooled serum and a medium and a high control from pooled serum supplemented with D(-)-lactic acid. Store frozen at -70°C, at which temperature these controls are stable for at least six months.

Procedure

Collect blood in either plain tubes or lithium heparin-containing tubes and separate the serum or plasma the same day. Set the rotor loader to deliver 60 µL of NAD+ reagent and 15 µL of working D(-)-lactate standard (cuvette no. 1) or patients' serum or plasma (cuvettes no. 2-20) together with 15 µL of wash water to each "sample" well, and 60 µL of D(-)-lactate dehydrogenase reagent and 30 µL of wash water to each "reagent" well. Allow any endogenous dehydrogenases and substrates from either in vivo pathological processes or from in vitro erythrocyte lysis (i.e., L(+)-lactate and L(+)-lactate dehydrogenase) to equilibrate with the NAD+ by preincubating the loaded rotor at 30 °C in the analyzer at the "incubate" speed (100 rpm for the Multistat III) for 20 min for mixing. Initiate the D(-)-lactate dehydrogenase reaction, using the following instrument settings: first filter, no. 1 (340 nm); second filter, no. 1 (340 nm); delay to first reading, 30 s; delay from first to second reading, 300 s; number of data points, 2; start mode, 1 (mixes contents of "sample" and "reagent" wells immediately on pressing the "start" button). The contents of the two wells are thus mixed by rapid acceleration and stopping and the D(-)-lactate concentrations calculated by comparing the increase in absorbance during the interval 30 s to 330 s after mixing for the 10 mmol/L aqueous standard and the specimens.

Results

The absorbance-concentration relationship is linear to about 12 mmol/L, after which there is a progressively larger underestimate of D(-)-lactate concentration: 18% low at 20 mmol/L and about 30% low at 30 mmol/L. Sera with values exceeding 12 mmol/L can be diluted with isotonic saline before analysis, the results being accurate for concentrations up to about 25 mmol/L, but this is rarely necessary because the clinically important range lies somewhere between 3 and 12 mmol/L (see below). Table 1 details between-day precision. Analytical recovery when 9.2 mmol of D(-)-lactate was added per liter to nine randomly selected sera was 99.5% (SD 3.4%, range 8.7 to 9.5 mmol/L). Grossly lipemic sera showed lower recoveries (50-90%) but pre-analysis ultracentrifugation at 140 000 × g resolved this problem. Icteric, hemolyzed, and slightly lipemic sera showed no interferences. Assays run on heparinized plasma and sera yielded essentially identical results. Sera before and after addition of as much as 50 mmol of L(+)-lactate per liter gave the same results for D-lactate concentration. This lack of response to L(+)-lactate was confirmed for each new lot of D(-)-lactate dehydrogenase. Sera collected from 28 healthy laboratory personnel showed a mean D(-)-lactate concentration of 100 mmol/L (SD 30 mmol/L, range 30 to 180 mmol/L). Note that the SD for the D(-)-lactate distribution in normal subjects is approximately the same as the within-

day SD for the low serum control pool, suggesting that most of the variation in the "normal" range comes from random analytical error.

Case study: A 47-year-old woman on whom a jejunoileal-bypass was done in 1976 reported repeated episodes over the last several years of disorientation, ataxia, and dysarthria, lasting for one to two days. During these episodes, laboratory results revealed a mild hyperchloremic metabolic acidosis with normal sodium and potassium, pH values of 7.15 to 7.20, chloride of 114 to 118 mmol/L, total carbon dioxide of 10 to 15 mmol/L, and anion gaps (calculated as [Na+] + [K+] - [Cl-] - [HCO3-]) of 15 to 20 mmol/L. Figure 1 shows her clinical symptoms and, when measured, the blood-gas results and D(-)-lactate concentrations for her recent "spells." D(-)-Lactate was not measured during the December 3rd episode, but on December 22nd, while she was asymptomatic, no D-lactate could be detected in her serum. On December 26th she again became disoriented, ataxic, and dysarthric, and the serum D(-)-lactate concentration was 8 mmol/L. The symptoms cleared within 8 h without therapy. On January 7th, she became stuporous and was treated with antibiotics and intravenous bicarbonate. On admission her serum D(-)-lactate concentration was 12 mmol/L, gradually declining over the next 24 h as her symptoms resolved.

Discussion

Apparently the clinical symptoms of D(-)-lactic acidosis begin to appear when the lactate concentration is about 4 mmol/L, severe symptoms (stupor, coma) appearing when the concentration is about 8 mmol of D(-)-lactate per liter (1, 6-9). The finding of a mean D(-)-lactate value of 0.1 mmol/L in healthy controls may be due to the metabolism of methyglyoxal to D(-)-lactate by the glyoxylate-1 system (11). The synthesis of methyglyoxal in mammalian tissues is at least partly non-enzymic, both during amino acid metabolism and from dihydroxyacetone phosphate (12, 13). Presumably D(-)-lactate is formed from methyglyoxal in human blood or other tissues where the glyoxylate system is active (14). However, the major source of D(-)-lactate in humans is anaerobic bacterial metabolism of pyruvate to D(-)-lactate. The catabolism of D(-)-lactate in mammals is unclear, although its urinary excretion (15) and metabolism to CO2 (16) have both been reported.

This method for D(-)-lactate analysis has proven to be

### Table 1. Between-Run Precision of Analysis for D-Lactate in 23 Pooled Control Sera

<table>
<thead>
<tr>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>D(-)-lactate mmmol/L</td>
<td>0.13</td>
<td>0.068</td>
</tr>
<tr>
<td>D(-)-lactate mmmol/L</td>
<td>1.24</td>
<td>0.060</td>
</tr>
<tr>
<td>D(-)-lactate mmmol/L</td>
<td>8.05</td>
<td>0.296</td>
</tr>
</tbody>
</table>

Fig. 1. Clinical symptoms and laboratory results in a patient with jejunoileal bypass, recurrent metabolic acidosis, and encephalopathy.
rapid, accurate, precise, and adaptable to routine clinical laboratory analysis. Because our method is automated it is substantially more convenient than former methods (3-5). Use of serum or plasma, rather than an ultratrate or protein-free supernate, and preincubation of specimen with NAD⁺ to eliminate interfering side reaction of endogenous dehydrogenases and their substrates simplifies the procedure. The working reagents are conveniently prepared from stock reagents with long-term stability. This assay should be useful in evaluating patients at risk of developing D(-)-lactic acidosis.

References

Salivary Urea Nitrogen as an Index to Renal Function: A Test-Strip Method

Toshihiro Akai,1 Kellichi Naka,1 Chikao Yoshikawa,1 Kiyoshi Okuda,1 Teruo Okamoto,2 Seiji Yamagami,3 Takashi Inoue,4 Yasuo Yamao,5 and Shigeki Yamada5

To investigate the feasibility of using salivary urea nitrogen as an index of renal glomerular filtration rate, we developed and applied a new analytical system consisting of a urease-containing test strip and an automatic reflectance spectrometer. The concentrations of urea nitrogen so determined correlate well \( r = 0.93 \) with concentrations in serum. These preliminary data suggest that our method can be used routinely as a simple and reliable means of detecting abnormalities of renal function.

Additional Keyphrases: urea nitrogen · glomerular filtration rate · kidney disease

Measurement of urea nitrogen in blood is valuable for diagnosing renal diseases, particularly those associated with a reduction in glomerular filtration rate (GFR). However, specimens are obtained by venipuncture, an invasive technique. We have developed an alternative, noninvasive technique, a dry-reactant test strip. Use of the strip is based on the observation (7) that the concentration of urea nitrogen in saliva reflects its concentration in serum so that one may validly substitute saliva for serum as the sample.

1 Department of Laboratory Medicine, 2 Second Department of Internal Medicine, 3 Department of Pediatrics, Osaka City University Medical School, Abeno, Osaka 543, Japan. 4 Inoue Hospital, Suita City, Osaka 564, Japan. 5 Kyoto Daiichi Kagaku Co. Ltd., Minamiku, Kyoto 601, Japan. Received April 12, 1983; accepted July 12, 1983.

Here we report our development of a simple, reliable system for measuring salivary urea nitrogen, and its application as an indicator of renal function.

Materials and Methods

Reagents. The reagent layer of the strip consists of chromatographic paper (no. 52; Toyo Chromatographic Paper Co., Ltd., Osaka, Japan) that was immersed in 1 mol/L Tris buffer (pH 8.5) containing 2 g of urease (EC 3.5.1.5, from jack beans; Toyobo Co., Ltd., Osaka, Japan) per liter. The indicator layer of the strip consists of cellulose acetate film that was soaked in acetone containing 1.6 g of bromcresol green (Wako Pure Chemical Industries Co., Ltd., Osaka, Japan) per liter.

In the urease–indophenol comparison method (2), we determined urea nitrogen in serum and saliva by using the "Urea NC Autotest" (Wako Pure Chemical Industries).

Instrument. An instrument that measures reflectance automatically was used. In this system, the intensity of a color developed on a test strip is measured as the reflectivity of a single wavelength of light (583 nm) by use of a spherical integrator.

Procedure. Moisten the reagent layer by touching it to the tongue or by applying one drop (about 50 μL) of serum. Then remove the strip, peel off the protective layer from an adhesive patch, and place the adhesive patch onto the exposed surface of the reagent layer containing the buffered urease (Figure 1).