


DOI: 10.1373/clinchem.2005.050472

Sensitive Spectrophotometric Assay for Plasma Oxalate, Paula M. Ladwig, Robert R. Liedtke, Timothy S. Larson, and John C. Lieske (*Department of Laboratory Medicine and Pathology and Division of Nephrology and Hypertension, Mayo Clinic College of Medicine, Rochester, MN; * address correspondence to this author at: Mayo Clinic, Division of Nephrology and Hypertension, 200 First Street SW, Rochester, MN 55905; fax 507-266-9315, e-mail Lieske.John@mayo.edu)

Precise measurement of plasma oxalate is difficult because the concentration in healthy humans is fairly low (1–3 μmol/L) (1, 2). A colorimetric enzymatic assay that uses oxalate oxidase is commonly used for oxalate detection (2, 3). This assay is fairly straightforward for detecting urinary oxalate, which occurs at concentrations in the millimolar range. Plasma oxalate, however, occurs at concentrations in the micromolar range, and signals generated by oxalate degradation are difficult to detect. Our reference laboratory previously used an oxalate oxidase–based assay that required enzyme immobilization on a nylon coil and uses an HPLC with spectrophotometric detection (1). This method has several disadvantages, including limited automation potential, and requires subjective estimation of peak size. We therefore took advantage of the enhanced sensitivity of currently available spectrophotometers (absorbance measured down to a sensitivity of 0.0001) to develop a plasma assay that uses soluble oxalate oxidase (1–4).

The reagents used were as follows: oxalate reagents A and B and the Oxalate Urine Control Elevated, purchased from Trinity Biotech; hydrochloric acid (0.01 and 12 mol/L), sodium hydroxide (10 mol/L), potassium citrate monohydrate (crystalline; formula weight 324.22), sodium hydroxide (10 mol/L), potassium citrate dihydrate (crystalline powder; formula weight 372.24), citric acid monohydrate (granular; formula weight 126.07), and oxalic acid dihydrate (crystalline powder; formula weight 126.07) from Sigma. Disposable polystyrene semimicro (1.5 mL) cuvettes were purchased through Fisher. Absorbance was measured with the Beckman Coulter DU800 UV/Visible Spectrophotometer.

Stock citrate buffer (0.33 mol/L) was prepared by dissolving 30.5 g of potassium citrate, 50 g of citric acid, and 2 g of EDTA disodium salt in 1 L of distilled, deionized H2O, with a mean (SD) resulting pH of 3.3 (0.2). Working citrate buffer (0.066 mol/L) was prepared from the stock and filtered through a 0.2 μm, 47-mm nylon filter just before use.

Oxalate stock solution (1.0 mmol/L) was prepared by dissolving 30.5 g of potassium citrate, 50 g of citric acid, and 2 g of EDTA disodium salt in 1 L of distilled, deionized H2O, with a mean (SD) resulting pH of 3.3 (0.2). Working citrate buffer (0.066 mol/L) was prepared from the stock and filtered through a 0.2 μm, 47-mm nylon filter just before use.
Blood from healthy volunteers was drawn in a 10-mL sodium heparin tube and immediately placed on wet ice. Within 1 h, samples were centrifuged at 4 °C to isolate plasma, which was adjusted within the next hour to a pH range of 2.3–2.7 with ~10 μL of concentrated (12 mol/L) HCl per 1.0 mL of plasma. Once acidified, samples were deproteinized by centrifugation at 1000 g at 20 °C for 1.5–2 h with an Amicon Ultra-4 filter. Sample filtrates (500 μL) controls, calibrators, and an HCl blank were next treated with sodium nitrite (30 μL of 5 mmol/L sodium nitrite in working citrate buffer) to convert sample ascorbate to dehydroascorbate.

Proper sample processing and acidification are essential (2, 5). Ascorbate converts to oxalate nonenzymatically at a pH >4.0; the higher the pH the more rapid the conversion (1). It is therefore necessary to maintain a low pH during sample processing. Deproteinization during subsequent steps is also required to prevent ascorbate precipitation, which can produce overwhelming turbidity, precluding spectrophotometric analysis, and trap unpredictable quantities of oxalate (5). Finally, recovery through the filters was found to be dependent on maintaining the pH in the desired range (2.3–2.7) (1).

Acidified, deproteinized, nitrite-treated plasma filtrates (100 μL), controls, calibrators, and HCl blanks were combined with 500 μL of Trinity Biotech oxalate reagent A [3.2 mmol/L 3-(dimethylamino)benzoic acid, 0.22 mmol/L 3-methyl-2-benzothiazolinone hydrazone along with buffer (pH 3.1) and nonreactive ingredients and stabilizers] in a cuvette, and the absorbance (~0.0400) was measured at 590 nm (A readings). Trinity Biotech oxalate reagent B [40 μL, containing 3000 U/L oxalate oxidase (barley) and 100 000 U/L peroxidase (horseradish)] was added, and the absorbance at 590 nm (approximate range, 0.0400–0.1500) was measured again (B readings). The A readings were subtracted from the B readings for each unknown, calibrator, control, and HCl blank. The subtracted HCl blank was then subtracted from each unknown, calibrator, and control. A calibration curve was generated to determine unknown values.

Control materials, calibrators, and a plasma pool were analyzed for intraassay precision (Table 1). Plasma was collected as described earlier, acidified, and pooled. This pool was separated into 10 aliquots and deproteinized. After nitrite treatment, each of the 10 aliquots was run in duplicate (total n = 20). Two concentrations of control material were analyzed (n = 20) over a 2-week period for interassay precision. Review of the data suggested that values as low as 1.0 μmol/L can be reliably detected with a CV ≤20%. Patient samples (n = 3) along with the 50 μmol/L calibrator were serially diluted (undiluted to 1:32 dilution) with nitrite-treated 0.01 mol/L HCl and assayed; the results demonstrated that the method is linear from 1 to 50 μmol/L (y = 0.9635x + 0.0918 μmol/L; r² = 0.9975; see the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol51/issue12/).

Previous studies suggested that ~15% of labeled oxalate is lost when the deproteinizing step is performed at pH 2.3–2.7 (1). Therefore, before performing the assay, we added increasing amounts of the 50 μmol/L oxalate calibrator to a normal plasma sample and pooled plasma samples (n = 3). The mean recovery of the added oxalate was 84%.

Oxalate oxidase is a specific enzyme for oxalate. There have been many interference studies done in the past that have looked at ~38 compounds, such as glyoxalate, glycolate, lactate, and pyridoxine (6–10). These com-

### Table 1. Precision and stability data for the proposed method for plasma oxalate.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean (SD), μmol/L</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intraassay precision</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 μmol/L calibrator</td>
<td>0.7 (0.19)</td>
<td>26</td>
</tr>
<tr>
<td>1.0 μmol/L calibrator</td>
<td>1.1 (0.15)</td>
<td>13</td>
</tr>
<tr>
<td>Low control</td>
<td>2.0 (0.26)</td>
<td>13</td>
</tr>
<tr>
<td>High control</td>
<td>20.9 (0.20)</td>
<td>1.0</td>
</tr>
<tr>
<td>Pooled plasma</td>
<td>4.3 (0.14)</td>
<td>3.3</td>
</tr>
<tr>
<td><strong>Interassay precision</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low control</td>
<td>2.2 (0.28)</td>
<td>13</td>
</tr>
<tr>
<td>High control</td>
<td>20.3 (0.39)</td>
<td>1.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stability</th>
<th>Difference, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidified unfiltered plasma</td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>4</td>
</tr>
<tr>
<td>Day 14</td>
<td>3</td>
</tr>
<tr>
<td><strong>Filtrate</strong></td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>2</td>
</tr>
<tr>
<td>Day 14</td>
<td>2</td>
</tr>
<tr>
<td>Day 21</td>
<td>8</td>
</tr>
<tr>
<td>Day 28</td>
<td>4</td>
</tr>
</tbody>
</table>

*Control material, calibrators, and a plasma pool were analyzed (n = 20) for intraassay precision. Two concentrations of control material were analyzed (n = 20) over a 2-week period for interassay precision. Review of the data suggests that values as low as 1.0 μmol/L can be reliably detected with a CV ≤20%. Stability studies were performed with acidified plasma and acidified deproteinized filtrate. Acidified deproteinized filtrate samples (n = 4) were stable when stored frozen for 28 days. Acidified plasma samples (n = 3) were also stable for at least 14 days when stored frozen (~20 °C).
pounds caused no interference. Ascorbic acid was found to be an inhibitor in concentrations >125 μmol/L (6).

We performed stability studies for acidified plasma and acidified deproteinized filtrate (Table 1) because plasma must be acidified immediately to avoid the nonenzymatic conversion of ascorbate to oxalate (1). Acidified, deproteinized, filtered samples (n = 4) were stable when stored frozen for 28 days. Acidified plasma samples (n = 3) were stable for at least 14 days when stored frozen (−20 °C). Because of the presence of denatured proteins, however, plasma that is acidified and frozen before deproteinization is extremely viscous when thawed, making further analysis difficult. Deproteinization of acidified samples before storage is therefore advisable.

Healthy volunteers (n = 102; 47 males and 55 females) were recruited through the Mayo Department of Laboratory Medicine and Pathology Quality Assurance Office. Mean donor age was 43 years (range, 22–76 years). Individuals with a history of kidney disease, nephrolithiasis, Crohn disease, gastric bypass or other gastrointestinal resection, or primary hyperoxaluria were excluded. Donors were requested to fast overnight and avoid vitamin C supplements for 24 h before the collection. For this adult population, the upper reference limit (95th percentile) of <1.8 μmol/L was established, which agrees with previous studies (1, 2). No variation with age or sex was apparent (Fig. 1).

Oxalate is a small, 2-carbon organic acid found in most plant tissues. It is thought that only ~10% of ingested oxalate is absorbed. Approximately one third of the oxalate in urine is from absorbed oxalate, and approximately two thirds is from oxalate synthesized by the liver (11). Oxalate intake has been reported to vary from 70 to 930 mg/day in a typical Western diet (12). Because humans have no enzymes to metabolize oxalate, it must be eliminated from the body via the kidneys (13).

Plasma oxalate concentrations are increased in patients with primary hyperoxaluria (14–17), an autosomal recessive disorder of glyoxalate metabolism characterized by excessive production and urinary excretion of oxalate resulting from defects in specific liver enzymes (alanine: glyoxalate transferase in type 1 and glycolate reductase in type 2). Accurate determination of plasma oxalate can be an important diagnostic test, particularly in young children, for whom collection of 24-h urine samples can be difficult, or in patients presenting in renal failure. In the latter group, a rapid and accurate diagnosis is particularly important because systemic oxalosis in primary hyperoxaluria patients causes them to do poorly on standard dialysis (18–20). Frequent determinations of oxalate and aggressive dialysis are necessary until kidney and/or liver transplantation can be performed (14). Plasma oxalate concentrations are increased in all patients with end-stage renal failure, regardless of the cause, although not to the extent seen in primary hyperoxaluria (21). Individuals with diverse gastrointestinal conditions that cause malabsorption often absorb oxalate from their diet (22–25), a condition termed enteric hyperoxaluria. Relatively little is known regarding the range of plasma oxalate concentrations observed in the enteric hyperoxaluric patient group, although oxalosis has been observed in some patients, associated with end-stage renal failure (23, 26). Therefore, accurate determination of plasma oxalate concentrations could be valuable for these enteric hyperoxaluric patients as well.

In conclusion, we describe a new, rapid, reliable spectrophotometric plasma oxalate assay that is less labor-intensive and technically demanding than our previously used assay. The Beckman Coulter DU800 spectrophotometer allows for enhanced sensitivity, down to 1 μmol/L, with a larger linear calibration curve and measuring range. The enhanced sensitivity of this method enables routine detection at the upper end of the reference interval with improved differentiation between results within and outside the reference interval.

References

Coenzyme Q₁₀ (CoQ₁₀), the predominant ubiquinone species in humans, functions as an electron carrier in the mitochondrial electron transport chain (ETC) and as an intracellular antioxidant (1). Although primary CoQ₁₀ deficiency is rare, a profound deficiency in skeletal muscle CoQ₁₀ has been reported in patients with multisystem mitochondrial encephalomyopathies (2, 3). Cardiovascular disease has been associated with a CoQ₁₀ deficiency (4, 5), and it is becoming increasingly apparent that other groups of patients may become CoQ₁₀ deficient, particularly individuals with ataxia (6) and some patients receiving statins (7).

When assessing tissue CoQ₁₀ status, we have found that the lack of a commercially available nonphysiologic internal standard (IS) is a major difficulty. Although naturally occurring ubiquinones have been used as ISs in this determination, they are not free from the influence of ubiquinones that might be present in human tissue as the result of dietary contamination (8) or synthesis by microorganisms (9, 10). There is a need, therefore, for an alternative IS that is not influenced by exogenous/endogenous ubiquinones. Di-ethoxy-CoQ₁₀ has been suggested as a nonphysiologic IS to determine CoQ₁₀ (11). In this study we evaluated this IS along with di-propoxy-CoQ₁₀ for their suitability to determine tissue CoQ₁₀. Reference intervals were established for the CoQ₁₀ concentration of skeletal muscle, blood mononuclear cells (MNCs), and plasma. A patient with a suspected CoQ₁₀ deficiency was subsequently identified.

Reference intervals were established for the following: (a) skeletal muscle from 26 patients [mean (SE) age, 24.5 (3.9) years; range, 0.5–59 years; ratio of males to females, 7:6] with no evidence of an ETC deficiency detected in their skeletal muscle biopsies; (b) MNCs from 17 healthy volunteers and 13 disease controls with no clinical evidence of an ETC deficiency [mean (SE) age, 32.6 (2.6) years; range, 1–61 years; ratio of males to females, 7:8]; and (c), plasma from 24 patients [mean (SE) age, 14.3 (2.9) years; range, 1–57 years; ratio of males to females, 2:1] with no clinical evidence of an ETC deficiency.

The correlation between skeletal muscle, MNC, and plasma CoQ₁₀ status was assessed in 2 groups of patients with no clinical or biochemical evidence of an ETC deficiency: Group 1 consisted of 12 patients [mean (SE) age, 13.21 (4.03) years; range, 1–43 years; male/female, 7:6] with no evidence of an ETC deficiency; Group 2 consisted of 14 patients [mean (SE) age, 24.5 (3.9) years; range, 0.5–59 years; ratio of males to females, 7:8]. Correlations between skeletal muscle and MNC CoQ₁₀ status and between skeletal muscle and plasma CoQ₁₀ status were determined with samples from group 1; correlations between MNC and plasma CoQ₁₀ status were determined with samples from groups 1 and 2.

The patient with a suspected CoQ₁₀ deficiency was a 47-year-old female, mentally retarded since birth, ataxic, and with poor vision and hypertrophic cardiomyopathy, in whom evidence of an ETC complex II–III (succinate cytochrome c reductase) deficiency [0.015; reference interval, 0.040–0.204 (activity expressed as a ratio to citrate synthase activity to allow for mitochondrial enrichment)] (12) had been detected in skeletal muscle.

MNCs were isolated from 5–10 mL of sodium EDTA-anticoagulated blood within 24 h of venesection by use of the ACCUSPIN™ system–Histopaque®-1077 (Sigma-Aldrich). The MNCs were suspended in phosphate-buffered saline (150 mmol/L NaCl, 150 mmol/L sodium phosphate), pH 7.2 (200 µL per 5 mL of blood), and stored at −70 °C until analysis. During this procedure, plasma was separated from the sodium EDTA-anticoagulated blood and stored at −70 °C until analysis.

Skeletal muscle biopsy homogenates were prepared as described by Heales et al. (12). Protein concentration was determined by the method of Lowry et al. (13). The synthesis of di-ethoxy-CoQ₁₀ was undertaken as de-