An Inexpensive Method for Sensitive Enzymatic Determination of Oxalate in Urine and Plasma

René J. Berckmans and Peter Boer

In this simple, sensitive, and rapid enzymatic method for the determination of oxalate in urine or plasma, oxalate oxidase (EC 1.2.3.4) prepared from barley seedlings is used to convert oxalate to carbon dioxide and hydrogen peroxide, which is determined photometrically at 600 nm, with use of horseradish peroxidase, by oxidative coupling of 3-methyl-2-benzothiazoline hydrzone with \( N,N \)-dimethylaniline. Plasma is pre-treated by ultrafiltration and co-precipitation of oxalate with calcium sulfate and ethanol, urine by dilution and reversed-phase chromatography on C18 columns. Analytical recovery for urine is 99 ± 2%, for plasma 92 ± 3%. The normal range for urinary excretion is 0.10 to 0.56 mmol/24 h, and for the concentration in plasma 0.4 to 3.7 \( \mu \)mol/L. There were no significant sex-related differences in urinary excretion or plasma concentration. Our within- and between-assay coefficients of variation were, respectively, <3.4% and <8.0% for urine, and <1.5% and <4.3% for plasma.

Additional Keyphrases: reference interval · oxalate oxidase · peroxidase · oxidative coupling · renal failure · primary hyperoxaluria

Through the years, many methods (1) have been published for determination of oxalate in urine or plasma, or both. Doesch (2) distinguishes the following groups of techniques: oximetric, colorimetric, enzymatic, atomic absorption spectroscopy, polarographic, isotope dilution, use of ion-specific electrodes, distribution chromatography, exchange- and gel-chromatography, and gas-chromatographic methods. Many of these techniques suffer drawbacks such as low analytical recovery, nonlinearity, use of radioisotopes, low specificity, too laborious, too insensitive, too expensive, or unreliable results. Some of these objections also apply to the procedures for oxalate in urine (3) and plasma (4) that we currently use. The method for urine has the disadvantages of low sensitivity, long incubation time (five days), and the use of \(^{14}C\) oxalate for correction of incomplete precipitation, while the method for plasma is laborious, also has a long incubation time (three days), and gives higher reference values than in vivo isotope clearance methods. Therefore we developed new methods.

Materials and Methods

Reagents

**Chemicals.** \(^{14}C\) Oxalic acid (specific acty. 2.74 TBq/mol) was purchased from the Radiochemical Centre, Amersham, U.K. \( N,N \)-Dimethylaniline was from Sigma Chemical Co., St. Louis, MO. Other chemicals ("p.a." grade) were from BDH Chemicals, Poole, U.K.

**Enzymes.** Oxalate oxidase (EC 1.2.3.4) was prepared from barley seedlings (4). The final solution contained 5 kU of oxalate oxidase per liter, specific activity 19 kU/g, and was stored in 3-mL portions at −20 °C.

Ascorbate oxidase (EC 1.10.3.3) from *Cucurbita* species was purchased from Boehringer, Mannheim, F.R.G. We dissolved 1000 U in 1 mL of demineralized water and stored the solution in 0.1-mL portions at −20 °C.

Peroxidase (EC 1.11.1.7) from horseradish (type VI) was purchased from Sigma Chemical Co. Four milligrams was dissolved in 25 mL of succinate buffer and stored in 2.5-mL portions at −20 °C.

**Solutions.** Succinate buffer (stored at 4 °C): succinic acid 50 mmol/L, EDTA disodium salt 5 mmol/L, thimerosal 6 \( \mu \)mol/L, pH adjusted to 3.8 with 1 mmol/L NaOH.

Cadmium sulfate dihydrate, 9.7 mmol/L.

Sodium oxalate (internal standard): 0.3 and 1.0 mmol/L in succinate buffer.

Color reagent: 125 \( \mu \)L of \( N,N \)-dimethylaniline, 5 mg of 3-methyl-2-benzothiazoline hydrzone hydrochloride, and 2.5 mL of peroxidase were dissolved in 100 mL of succinate buffer immediately before use and shielded from light with aluminum foil.

**Equipment.** Absorbance was measured with an RRP photometer (Vitatron, Dieren, Netherlands); pH with a PHM 62 pH meter (Radiometer, Copenhagen, Denmark) with an Amagrus C-1016 electrode (Russell, Helmstadt, F.R.G.); reaction rates with a PU-8800 spectrophotometer (Phillips, Eindhoven, Netherlands). For reversed-phase chromatography we used a Vac-Elut vacuum extraction system (Analytichem, Harbor City, CA) and Bond Elut octadecyl silane bonded silica C18 columns containing 200 mg of sorbent (Analytichem). For ultrafiltration we used Centriflo CF-25 membrane cones (Amicon, Danvers, MA), washed with distilled water and centrifuged for 15 min at 1000 × g before use.

**Procedure**

**Sampling.** Heparinized blood was processed within 4 h after collection. Plasma was stored at −20 °C for not longer than five days. Oxalate concentrations remain stable for seven days at −20 °C and for 100 days at −70 °C.

Twenty-four-hour urine specimens were collected in bottles containing 10 mL of concentrated hydrochloric acid. The oxalate concentrations remain stable for at least six months at −20 °C.

**Pre-treatment.** Wash C18 columns once with ammonia (10 mol/L)/methanol (1:99 by vol), twice with methanol, and twice with distilled water.

Plasma: Mix 4.75 mL of plasma and either 0.25 mL of succinate buffer or internal oxalate standard (0.3 mmol/L), pour into an Amicon cone, and centrifuge for 1 h (4 °C, 1000 × g). Mix 1.0 mL of filtrate (in triplicate) in plastic conical tubes with 0.5 mL of 9.7 mmol/L CaSO₄ and 3 mL of absolute ethanol. After at least 2 h of precipitation, centrifuge the mixture at 3000 × g. Aspirate the supernate.

Urine: Dilute 1.0 mL of acidified urine with 4.0 mL of succinate buffer. Mix 1.8 mL of this diluted urine with 0.2 mL of either succinate buffer or internal oxalate standard (1.0 mmol/L). Apply 1 mL of this solution onto a C18 column and collect the effluent in a plastic tube. Wash the column with 10 mL of 9.7 mmol/L CaSO₄ and 3 mL of absolute ethanol. After at least 2 h of precipitation, centrifuge the mixture at 3000 × g. Aspirate the supernate.

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with 1.0 mL of succinate buffer and 0.1 mL of hexane to expel the water from the column. Combine the aqueous phases.

**Color reaction.** Dissolve the plasma precipitates (in triplicate) in 1.5 mL of color reagent. Mix 0.2-mL aliquots of urine extracts (in triplicate) with 1.3 mL of color reagent. After a 5-min pre-incubation, add 20 μL of oxalate oxidase to two of the three tubes. Use the other tube as a blank. After mixing their contents, incubate the tubes at 37 °C for 30 min. Measure the resulting color at 600 nm.

**Calculations.** Extinction differences:

A = reagent blank with oxalate oxidase - reagent blank without oxalate oxidase.

B = sample - sample blank.

C = sample with internal standard - sample blank with internal standard.

Final concentration of internal standard in diluted urine 0.10 mmol/L; in plasma 15 μmol/L.

Calculation of urinary excretion of oxalate:

\[
\frac{B - A}{C - B} \times \frac{2.0}{1.8} \times 0.10 \times 24-h \text{ urine vol (L)} = \text{mmol/24 h}
\]

Calculation of oxalate concentration in plasma:

\[
\frac{B - A}{C - B} \times \frac{5.0}{4.75} \times 15 = \mu mol/L
\]

**Previous methods.** We compared results by these newly developed methods with those by the methods we previously used, for samples from healthy subjects and from patients with renal diseases, including diabasis patients.

The previous methods were as follows. Urinary oxalate (3) was determined after prolonged precipitation with calcium chloride in the cold. Corrections for incomplete precipitation were made by addition of [14C]oxalate. Oxalate was measured by an indirect colorimetric method based on the decrease in absorbance of the red uranium(IV)-4-(2-pyridylazo)resorcinol complex caused by oxalate. Plasma oxalate (4) was measured in deproteinized and acidified plasma by enzymatic conversion to carbon dioxide and hydrogen peroxide with oxalate oxidase. The decrease in pH of a dilute carbonate–bicarbonate buffer was used as a measure of the amount of carbon dioxide liberated by the enzyme.

**Results**

**Analytical Variables**

**Linearity.** The relation between analyte concentration and color intensity was evaluated for water, urine, and plasma. The relation was linear up to an absorbance (A) of 0.800. The upper concentration limit for linearity is 1.00 mmol/L for urine and 20 μmol/L for plasma. Samples with higher concentrations must be diluted and re-assayed.

**Color development time.** The color development of the indamine dye was monitored for 2 h in water, urine, and plasma. In all cases the reaction was complete by 20 min (Figure 1).

**Detection limit.** The detection limit, defined as the concentration which gives a difference in absorbance twice the standard deviation of the reagent blank, was 0.08 μmol/L for both urine and plasma.

**Analytical recovery.** Recovery was evaluated after adding 60 Bq of [14C]oxalate to urine or plasma before pre-treatment, both to samples with and without added unlabeled oxalate (urine 0.1 mmol/L, plasma 50 μmol/L). The recovery values for urine with and without addition of unlabeled oxalate (n = 10) were 98 ± 3% and 99 ± 2% (P > 0.05) and for plasma (n = 10) 90 ± 3% and 91 ± 2% (P > 0.05), respectively.

**Precipitation time for plasma.** The precipitation time for plasma was evaluated by adding 60 Bq of [14C]oxalate, followed by measurement after 2, 4, 6, and 24 h of precipitation. Mass analytical recoveries were 82% (SD 3%), 83% (SD 3%), 86% (SD 3%), and 85% (SD 3%), respectively. These values are not significantly different by analysis of variance and the studentized Newman–Keuls test (P > 0.05).

**Dilution of urine.** Processing of undiluted urine resulted in incomplete color development of the indamine dye. Color development was maximal when urine was diluted at least fivefold (Figure 2).

**Sample stability and storage.** Processing of blood or heparinized plasma up to 4 h after collection had no significant effect on the measured concentration of oxalate in plasma, whether stored at 4 °C or 25 °C (Table 1). For samples stored at −20 °C, the value obtained for oxalate remains stable for seven days for plasma, five days for ultrafiltrate. Longer storage times resulted in up to a fourfold increase in values after three months. Stored at −70 °C, samples could be used for as long as three months. Oxalate in acidified urine (pH < 2) stored at −20 °C remained stable for at least six months.

**Inhibition and activation.** Other authors (5–9) have already studied 38 compounds for possible interferences. We
Table 1. Effect of Delayed Processing of Blood and Plasma on Plasma Oxalate (μmol/L), after Storage at 4 °C and 25 °C (n = 3)

<table>
<thead>
<tr>
<th>Time, h</th>
<th>4 °C</th>
<th>25 °C</th>
<th>4 °C</th>
<th>25 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.2</td>
<td>2.2</td>
<td>2.2</td>
<td>2.2</td>
</tr>
<tr>
<td>1</td>
<td>2.0</td>
<td>2.2</td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td>2</td>
<td>2.1</td>
<td>2.3</td>
<td>2.0</td>
<td>2.2</td>
</tr>
<tr>
<td>4</td>
<td>2.0</td>
<td>1.8</td>
<td>2.0</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Table 2. Effect of Added Ascorbate Oxidase on Measured Oxalate Concentration (mmol/L) in Urine Supplemented with Ascorbic Acid

<table>
<thead>
<tr>
<th>Ascorbic acid added, mmol/L</th>
<th>Without ascorbate oxidase</th>
<th>With ascorbate oxidase</th>
<th>Incubated 60 min with ascorbate oxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.15</td>
<td>0.17</td>
<td>0.15</td>
</tr>
<tr>
<td>1</td>
<td>0.0</td>
<td>0.14</td>
<td>0.16</td>
</tr>
<tr>
<td>2</td>
<td>0.0</td>
<td>0.15</td>
<td>0.17</td>
</tr>
</tbody>
</table>

We reassessed the effect of ascorbic acid (0.125, 0.25, 0.5, and 1.0 mmol/L), glyoxalic acid and glycolate (1, 2, 4, 8, and 16 mmol/L), pyridoxine and thiamine (0.2, 0.4, 0.8, and 1.6 μmol/L), and nitrate (0.75, 1.5, 3.0, and 6.0 mmol/L), all dissolved in urine and processed as described under Methods. Ascorbic acid inhibited in concentrations >125 μmol/L. A slight inhibition was seen with glyoxalic acid in concentrations exceeding 4 mmol/L. The other compounds caused no interference.

We assessed the effect of removal of ascorbic acid with sodium nitrite (10) and ascorbate oxidase (11). Four randomly selected urine samples with ascorbic acid concentrations of 0, 1, and 2 mmol/L were processed as described under Methods.

Removal with NaNO₂: 10 μL of 100 mmol/L NaNO₂; 0.2 mL of extract (see urine pre-treatment). A yellow color appeared both in samples and blanks, probably as the result of disturbance by NaNO₂.

Removal with ascorbate oxidase: (a) as described by Crawford et al. (11): 10 μL of enzyme; 0.2 mL of extract; 60-min pre-incubation followed by color reaction, (b) same procedure without the 60-min pre-incubation. The results are given in Table 2.

Comparison of Methods

We compared the methods presented in this paper with the methods we were using (3, 4), conducting the comparison as recommended by Bland and Altman (12) and Westgard and Hunt (13). Our data on urine (n = 40) are shown in Figure 3. The means for the methods were 0.25 ± 0.10 and 0.24 ± 0.09 mmol/L, respectively, with a mean difference (D) of +0.01 (P > 0.05, Student's t-test). The regression equation is y = 0.870x + 0.222, with a correlation coefficient of 0.95 (P < 0.001). Limits of agreement (D ± 2SD) were −0.07 to 0.05 mmol/L (range of measurements 0.07 to 0.44 mmol/L); the fractional error was 0.038 ± 0.14.

Our data on plasma (n = 20) are presented in Figure 4. The mean for the new method was 28 ± 23 μmol/L for the old one 56 ± 43 μmol/L. The mean difference was −28 (P < 0.02); the regression equation is y = 1.812x + 4.305, with a correlation coefficient of 0.96 (P < 0.001). Limits of agreement were −18 to 73 μmol/L (range of measurements 2.8 to 154.0 μmol/L); the fractional error was −0.49 ± 0.17.

Coefficients of Variation

The within- and between-assay CVs are given in Table 3.

Reference Values

Reference values for oxalate in urine and plasma were obtained from data on healthy subjects who had been on an oxalate-restricted diet (14) for two days. We compared the method presented in this paper with other published methods in which oxalate oxidase in combination with 3-methyl-2-benzothiazoline hydrazine/N,N-dimethylaminoc POL (or 3-di-methylaminobenzoic acid) was used (Table 4). We found no significant sex-related differences.

Table 3. Within- and Between-Assay Coefficients of Variation for Determination of Oxalate in Urine and Plasma

<table>
<thead>
<tr>
<th></th>
<th>Urine</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean, mmol/L</td>
<td>CV, %</td>
</tr>
<tr>
<td>Within-assay</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.56</td>
<td>3.2</td>
</tr>
<tr>
<td>6</td>
<td>0.30</td>
<td>3.4</td>
</tr>
<tr>
<td>Between-assay</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>0.58</td>
<td>4.5</td>
</tr>
<tr>
<td>14</td>
<td>0.25</td>
<td>6.0</td>
</tr>
</tbody>
</table>
Table 4. Reported Normal Reference Values for Urine and Plasma Oxalate with Techniques Involving Oxalate Oxidase and 3-Methyl-2-benzothiazoline Hydrazine/N,N-Dimethylaniline (or 3-Dimethylaminobenzalcohol Acid) 

<table>
<thead>
<tr>
<th>Year (mmol/24 h)</th>
<th>Range</th>
<th>Mean</th>
<th>n</th>
<th>Pre-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1980</td>
<td>0.31–0.48</td>
<td>0.42</td>
<td>4</td>
<td>Ion-exchange/charcoal</td>
</tr>
<tr>
<td>1983</td>
<td>0.25–0.51</td>
<td>0.38</td>
<td>23</td>
<td>Ion-exchange/FeCl3</td>
</tr>
<tr>
<td>1983</td>
<td>0.16–0.56</td>
<td>0.36</td>
<td>97</td>
<td>Precipitation (CaSO4/ethanol)</td>
</tr>
<tr>
<td>1984</td>
<td>0.06–0.35</td>
<td>0.20</td>
<td>67</td>
<td>Extraction with Al2O3</td>
</tr>
<tr>
<td>1986</td>
<td>0.02–0.40</td>
<td>0.21</td>
<td>42</td>
<td>Extraction with Al2O3</td>
</tr>
<tr>
<td>1987</td>
<td>0.10–0.56</td>
<td>0.28</td>
<td>27</td>
<td>Dilution and reversed-phase chromatography</td>
</tr>
<tr>
<td>1983</td>
<td>0.21–0.71</td>
<td>0.41</td>
<td>50</td>
<td>Precipitation with CaCO3, with isotope correction and colorimetry</td>
</tr>
</tbody>
</table>

Plasma (mmol/L) 

<table>
<thead>
<tr>
<th>Year (n)</th>
<th>Range</th>
<th>Mean</th>
<th>n</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>1980</td>
<td>7.46</td>
<td>26.5</td>
<td>50</td>
<td>Directly</td>
</tr>
<tr>
<td>1983</td>
<td>7.22</td>
<td>15.8</td>
<td>15</td>
<td>Directly</td>
</tr>
<tr>
<td>1983</td>
<td>7.15</td>
<td>11.5</td>
<td>15</td>
<td>Directly</td>
</tr>
<tr>
<td>1985</td>
<td>4.22</td>
<td>13.0</td>
<td>36</td>
<td>Deproteinization and extr. of lipids</td>
</tr>
<tr>
<td>1985</td>
<td>&lt;3.5</td>
<td>8</td>
<td>Ultrafiltration</td>
<td></td>
</tr>
<tr>
<td>1986</td>
<td>1.3–3.1</td>
<td>2.0</td>
<td>21</td>
<td>NaNO2/ultrafiltration</td>
</tr>
<tr>
<td>1986</td>
<td>0.55</td>
<td>27.5</td>
<td>28</td>
<td>Alk. ultrafiltration</td>
</tr>
<tr>
<td>1987</td>
<td>0.4–3.7</td>
<td>2.0</td>
<td>49</td>
<td>Ultrafiltration/pipn</td>
</tr>
<tr>
<td>1984</td>
<td>1.2–6.4</td>
<td>3.3</td>
<td>24</td>
<td>Deproteinization and manometry</td>
</tr>
</tbody>
</table>

Discussion

In our department, a simple procedure is needed for measurement of oxalate in urine and plasma without use of special expensive equipment, for investigations of patients with renal failure and primary hyperoxaluria. The present method fulfills this criterion. It can be applied to plasma and urine samples, but a different pre-treatment is necessary, because urine and plasma contain different compounds disturbing the end-reaction. For urine, reversed-phase C18 chromatography was found to be a good approach to remove inhibitory compounds. Other methods, such as precipitation with CaSO4/ethanol (7), extraction with Al2O3 (8), and dilution (24), were unsatisfactory in our hands. Urine samples are diluted at least fivefold, to obtain maximal recovery. In the case of plasma, deproteinization by ultrafiltration followed by co-precipitation with CaSO4/ethanol eliminated inhibitory disturbances. Direct measurement in plasma as described by Sugiura et al. (15) was unsatisfactory because of very low sensitivity.

Although we seldom encountered interference by ascorbate or inhibitory compounds (20, 21), we added oxalate as internal standard to each sample as a precaution. Ascorbate interferes only at very high, non-physiological concentrations, which can be eliminated by use of ascorbate oxidase. Kasidas and Rose (22) recommended sodium nitrite for removing ascorbic acid, but this caused a yellow color that interfered in our determination.

These new methods are considerably less time consuming than the ones we previously used (3, 4). Twenty urine samples can be processed in half a working day (old method: 15 samples in six days, real handling time two half-days). For the new plasma determination these figures are 12 samples in one day (old method: four samples in four days, real handling time two half-days). Comparison of results for the urine methods showed no significant difference between the determinations. The normal reference interval was in the same range as reported in the literature (3, 7). The concentrations in plasma found with the new method were about twofold lower than with the old one and approached the values obtained with in vivo isotope determinations, which may be considered as "true" values (23). This suggests that the new method is superior to the one we previously used.

References