Spectrophotometric Determination of Urinary Oxalate with Oxalate Oxidase Prepared from Moss

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A novel spectrophotometric enzymic procedure for estimating oxalic acid in urine is described. Oxalate oxidase, prepared from moss species, converts oxalic acid to hydrogen peroxide and carbon dioxide. Hydrogen peroxide is determined enzymatically with horseradish peroxidase, by oxidative coupling of 3-methyl-2-benzothiazolinone hydrazone with N,N-dimethylaniline; the resulting indamine dye is determined spectrophotometrically at 595 nm. Interfering substances are removed by adsorption to ion-exchange resins and oxidation with charcoal, thus avoiding oxalate recovery problems accompanying oxalate isolation. The procedure is rapid, sensitive, linear, and precise. Results agreed well with those obtained with a widely used chemical technique.

Additional Keyphrases: enzymic methods • spectrophotometry • reference intervals

Measurement of oxalic acid in urine is of clinical interest in two groups of patients: those with urinary tract stones, and those with conditions in which increased excretion of oxalate is known to occur (primary and secondary hyperoxaluric syndromes). Although many measurement procedures have been described, few are accurate, precise, yet simple enough for the analysis of large numbers of specimens (1). Most methods for urinary oxalate involve two steps: isolation of oxalate from interfering substances by precipitation, extraction, or ion-exchange adsorption, followed by estimation of oxalate or a derivative using volumetric titration, colorimetry, fluorometry, atomic absorption spectroscopy, or gas-liquid chromatography. There is often incomplete recovery because of losses in the separation stages, which necessitates the use of a radioactive internal standard to serve as a recovery marker. Enzymic techniques have been described that do not involve a preliminary separation, but the reported methods have various disadvantages. Oxalate decarboxylase (EC 4.1.1.2) converts oxalate to carbon dioxide and formic acid. The carbon dioxide may be trapped by diffusion in a buffer and quantitated by the change in pH directly or color of a suitable indicator. However, such diffusion-dependent assays are time consuming (2–5). Alternatively, the formate may be quantitated by using a second enzyme, formate dehydrogenase (EC 1.2.1.2). This method is difficult in practice, because the enzyme requires NAD⁺ as a cofactor and commercially available NAD⁺ is often contaminated with formic acid (6, 7). A further disadvantage is that the enzymic steps cannot be carried out in a single-stage reaction, since the two enzymes have widely differing pH optima.

The present procedure involves the use of oxalate oxidase (oxalate:oxygen oxidoreductase, EC 1.2.3.4). This enzyme, which occurs in moss (8) and barley roots (9, 10), produces two moles of carbon dioxide and one mole of hydrogen peroxide from one mole of oxalic acid. The hydrogen peroxide generated from oxalate breakdown is quantitated by measuring the absorbance of chromophore formed by horseradish peroxidase (EC 1.11.1.7) catalyzed oxidation of a chromogen. Interfering substances, particularly divalent metals and ascorbic acid, are removed by ion-exchange chromatography and charcoal oxidation. While this work was in progress, a report appeared in which oxalate oxidase was used to quantitate urinary oxalate by measuring evolved carbon dioxide (11).

Materials and Methods

Enzyme. Oxalate oxidase was prepared from several species of moss (Mnium menziesii, Mnium insigne, Mnium ovale, Hylocomium splendens, Eurohynchium stokesii, and Rhytidadelphus triquetrus), which were collected on field trips in Washington by B.J.D.M. After collection, the moss leaves were removed with fine scissors, washed, dried at room temperature, powdered in a Wiley Mill or mortar and pestle, and stored at 4 °C. Material treated in this way retained enzyme activity for at least three months. Powered moss (0.3 g) was homogenized with 20 mL of 1 mol/L sodium chloride by using a 40 mL glass homogenizer (Wheaton Scientific, Millville, NJ 08332). The homogenate was centrifuged at 10 000 x g for 30 min (Sorvall Superspeed RC2-B Centrifuge; Ivan Sorvall Inc., Newton, CT 06470). The supernatant fraction was spun at 59 000 x g for 60 min (Beckman L Ultracentrifuge; Beckman Instruments Inc., Fullerton, CA 92634), then decanted, and the second supernate was dialyzed against several changes of distilled water for 24 h. Homogenization, centrifugation, and dialysis were performed at 4 °C. The dialyzed preparation was lyophilized and stored at 4 °C. Enzyme required for analytical use was prepared daily by dissolving the required amount in sodium phosphate buffer (50 mmol/L, pH 7.1), 10 mg of lyophilized powder in 1 mL of buffer.

The activities of the preparations varied with the species of moss. Activities of 50 U/L were obtained from Mnium species, and 25 U/L for Hylocomium splendens, and Eurohynchium stokesii, all of which were suitable for analytical purposes (one unit of enzyme catalyzing the transformation of one micromole of substrate per minute). The activity of Rhytidadelphus triquetrus preparations (10 U/L) was too low for analytical use.

Three grams of moss powder from Mnium species produced sufficient enzyme for 90 to 110 analyses.

Color reagent. The chromogen described by Gochman and Schmitz (12, 13) was used (Figure 1). Stock solutions of 3-methyl-2-benzothiazolinone hydrazone (MBTH; Eastman Organic Chemicals, Rochester, NY 14650; 1 g/L) and N,N-dimethylaniline (DMA; Fisher Scientific Co., Fair Lawn, NJ 07410; 2.5 g/L) were prepared in 0.1 mol/L hydrochloric acid. These were stored in amber-colored glass bottles at 4 °C. Working reagent was prepared fresh daily by diluting 2.5 mL

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of stock MBTH solution, 5.0 mL of stock DMA solution, and 0.6 mL (1500 U) of horseradish peroxidase (Grade 1 suspension in ammonium sulfate; Boehringer Mannheim, Indianapolis, IN 46250) to 100 mL with citrate buffer (50 mmol/L, pH 4.0). This "combined reagent" was shielded from light and kept at 4 °C until used.

Standards. We prepared 0.5, 1.0, and 2.0 mmol/L standard solutions of oxalic acid dihydrate in 0.1 mol/L hydrochloric acid and stored them at 4 °C.

Analytical procedure. Columns and resins for ion-exchange chromatography were supplied by Bio-Rad Laboratories, Richmond, CA 94804. Columns were fitted with two-way tape, to control flow. Nine milliliters of urine or standard was passed through columns containing Chelex-100 (Na⁺ form) and AG-50 (H⁺ form), as shown in Figure 2. The effluent was discarded; a further 1.5-mL sample was applied to the column, and the effluent was collected. Aliquots (0.5 mL) of each sample were transferred to a glass tube and mixed with 25 μL of 6 mol/L sodium hydroxide. One spatula point of charcoal (approximately 10 mg of "Norit" grade, Fisher Scientific Co.) was added to each tube. After mixing, the tubes were centrifuged and 25 μL of supernate was removed. This was mixed with 2.0 mL of the combined color reagent and 100 μL of oxalate oxidase preparation was added, giving a mixture with pH 4.1. The samples were incubated at room temperature in the dark for 60 min, following which the absorbance was measured at 595 nm and 25 °C in a Stasar III Spectrophotometer (Gilford Laboratories, Inc., Oberlin, OH 44074). Standards and blanks were included in each batch, all samples being treated in the same manner. Blanks were included for each sample in which the oxalate oxidase was omitted and in which water was substituted for the sample. Samples were analyzed in duplicate

Results

With different batches of enzyme the time of maximum color development varied between 15 and 50 min. The absorbance with 1.00 mmol/L oxalate standards was identical to that found when 1.00 mmol/L hydrogen peroxide was used, indicating the formation of peroxide to be stoichiometric. Although catalase may be present (8), it does not interfere at the pH used. If samples were shielded from the light, the color developed was stable for more than 3 h.

The linearity of the assay was tested by adding known amounts of oxalic acid to urine. The results (Figure 3) demonstrate that concentration and absorbance are linearly related up to 2.00 mmol/L (18 mg of anhydrous acid per 100 mL). This includes concentrations found in normal and most hyperoxaluric specimens. The intercept on the y-axis and the absorbance for untreated urine both represent the absorbance due to naturally occurring oxalic acid, the values coinciding. Oxalate concentrations as low as 50 μmol/L could be measured.

Overall recovery for five different oxalate concentrations is shown in Table 1. The range was 95–109%, with a mean value of 100%. Recovery of [14C]oxalic acid (New England Nuclear, Boston, MA 02118) added to 1.0 mmol oxalic acid solutions was 100% from the cation-exchange columns. Charcoal affected the recovery of oxalic acid from acid and neutral solutions. Recovery was 56.7% at pH 1.0 and 83.3% at pH 7.0. In alkaline solution (pH 13.0) oxalate recovery was 98.3%.

The intra-assay coefficient of variation was 11.0% at a urinary concentration of 0.25 mmol/L (2.3 mg of anhydrous oxalate per 100 mL), and 1.4% at an oxalate concentration of 1.39 mmol/L (12.5 mg/100 mL). Ten samples were analyzed in each
The amount determined has been corrected for the urinary oxalic acid already present. Each value represents the mean of duplicate analyses.

The effect of possible interfering substances was investigated by assaying 25 μL of 1.00 mmol/L oxalic acid in the presence of 25 μL of 5.0 mmol/L solutions of test substance, and determining the apparent concentration of oxalate. The substances tested were acetyl-salicylic acid, ascorbic acid, cysteine, dihydroxyphenylacetic acid, dihydroxyphenylalanine, fructose, fumaric acid, glucose, glutaric acid, glycolic acid, glyoxylic acid, L-lactic acid, maleic acid, malonic acid, oxaloacetic acid, pyruvic acid, succinic acid, tartric acid, and uric acid. Apparent oxalate concentrations of 1.00 mmol/L were found with all substances other than ascorbic acid (apparent oxalate concentrations < 0.1 mmol/L), cysteine (0.8 mmol/L), and dihydroxyphenylacetic acid (0.5 mmol/L). The negative interference due to 5.0 mmol/L cysteine and dihydroxyphenylacetic acid, but not that due to 5.0 mmol/L ascorbic acid, was eliminated by the ion-exchange/charcoal stages. The formation of chromophore was inhibited by ascorbic acid if oxalate oxidase was omitted, indicating that the inference is with the peroxidase-mediated reaction. The amount of charcoal used eliminated interference caused by ascorbate in concentrations up to 2.8 mmol/L (50 mg/100 mL). Because urine samples with ascorbate exceeding 2.8 mmol/L gave a positive result with Clinistest (Ames Co., Miles Laboratories Inc., Elkart, IN 46514), this test was performed on each sample. When positive, the ascorbate in urine was quantitated by 2,6-dichlorophenol titration. When ascorbate values exceeding 2.8 mmol/L were found, the patient was instructed to discontinue taking vitamin supplements for 48 h before a repeat urine collection.

To determine the accuracy of the method, we compared the present procedure with the existing assay in use in our laboratory. The existing technique (14) involved extraction of urine with ether/ethanol, back extraction into an alkaline aqueous phase, calcium precipitation, reduction of oxalate to glycolate, and separation of glycolate by ion exchange, followed by colorimetric determination with 2,7-dihydroxyazaphthalene and concentrated sulfuric acid. Losses were corrected by the recovery of an added radioactive internal standard. This procedure was highly specific, but tedious and cumbersome.

Thirty-one samples were analyzed by both techniques; Figure 4 shows the results, expressed as output of oxalic acid (milligrams of anhydrous acid per day). The slope of the calculated regression line was 0.96; the y-intercept was 0.27; the correlation coefficient was 0.90.

Daily oxalate excretion was measured in four healthy adult male subjects, no dietary restrictions being imposed. Values of 28, 38, 42, and 43 mg/day were obtained. Normal ranges for oxalate excretion found in previous studies in which similar chemical techniques were used are shown in Table 2.

![Fig. 4. Correlation between enzymic (oxalate oxidase) technique and chemical (extraction/reduction/colorimetry/isotope dilution) procedure](image)

The calculated regression (solid) and 45° (dotted) lines are shown. The regression equation is \( y = 0.96x + 0.27 \), \( r = 0.90 \)

**Discussion**

Our enzymic procedure has been developed for the determination of oxalic acid in urine by linking oxalate oxidase with horseradish peroxidase. This is possible because horseradish peroxidase contains an isoenzyme with activity at acid pH (18, 20). The assay is precise, sensitive, and rapid, particularly when compared with chemical methods (e.g., 14, 16) or enzyme techniques that rely on carbon dioxide diffusion (2-5, 11). Because overall recoveries average 100%, isotope dilution correction procedures are unnecessary.

Oxalate oxidase is inhibited by divalent metals and is sensitive to changes in the concentration of sodium chloride (9, 10). These potential interferences are removed by ion exchange. No other carboxylic acid tested interferes with the assay. Although ascorbic acid interferes, low concentrations of it are destroyed by charcoal and higher concentrations are detected by testing for reducing substances. Drug interferences are guarded against by including enzyme blanks.

The normal-range studies reported here are limited, but the values obtained agree well with ranges reported in studies in which a chemical procedure was used similar to that used for

<table>
<thead>
<tr>
<th>Table 2. Values for Urinary Oxalate Excretion by Healthy Subjects as Measured by Chemical Methods Similar to That Used in the Present Study for Correlation with the Enzymic Procedure</th>
<th>Author (ref.)</th>
<th>Subjects</th>
<th>Oxalate output, mg/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dempsey et al., 1960 (15)</td>
<td>33 collections</td>
<td>15-50</td>
<td></td>
</tr>
<tr>
<td>Hodgkinson &amp; Williams, 1972 (16)</td>
<td>22 adults</td>
<td>17-47</td>
<td></td>
</tr>
<tr>
<td>Earnest et al., 1974 (14)</td>
<td>20</td>
<td>44 ± 5 (SEM)</td>
<td></td>
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<tr>
<td>Dobbs and Binder, 1977 (17)</td>
<td>15</td>
<td>5-45</td>
<td></td>
</tr>
<tr>
<td>Stauffer, 1977 (18)</td>
<td>7</td>
<td>46.5 ± 1.54 (SEM)</td>
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the correlation study (Table 2). There is little reason to suppose that a more extensive investigation of reference interval would yield significantly differing results. However, a study is required in which the effect of diet, age, and sex and race of the subject on oxalate output is investigated. Such a study is considered outside the scope of the present paper, because oxalate output is affected by environmental factors, particularly diet.

The major disadvantage of the assay is that at present there is no suitable commercial preparation of oxalate oxidase available. However, moss may be gathered on field trips and possibly cultured in the laboratory. The method of preparation is more simple than that described for oxalate oxidase from barley roots (10). Enzyme from this source has been used to measure oxalate by quantitation of carbon dioxide production (11) and may prove suitable for quantitation by measuring peroxide formation.

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References