A Radioenzymatic Isotope-Dilution Assay for Oxalate in Serum or Plasma

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This assay for oxalate in serum or plasma, by a radioenzymatic isotope dilution technique, is sensitive, specific, and accurate. The oxalate in 5 mL of serum or plasma ultrafiltrate is precipitated with Cs2+, dissolved in acid, extracted with ether, and the extract evaporated under nitrogen and redissolved in citrate buffer. The oxalate in this solution is decarboxylated with oxalate decarboxylase (EC 4.1.1.2) in the presence of [14C]oxalate to evolve labeled and unlabeled CO2. An equation derived by Newsholme and Taylor [Biochim. Biophys. Acta 158, 11 (1968)] described quantitatively the simultaneous effects of variation of non-radioactive substrate on isotope dilution and enzyme velocity, resulting in a linear standard curve. We found the Km for this enzyme to be 31.0 μmol/L, with an assay sensitivity of 0.5 μg and a linear assay range to 10 μg. The sensitivity of this result was consistent with the value of 0.1 Km predicted from the equations of Newsholme and Taylor. The Kf values for phosphate and sulfate, two commonly occurring inhibitors of the enzyme that affect assay sensitivity by the relationship Km (1 + 1/Kf), were 12 and 7 mmol/L, respectively. Analytical recovery of 0.5–2.0 mg of unlabeled oxalate added to serum per liter was 95.4 ± 5.7% (SE). Inter- and intra-assay precision was excellent (coefficients of variation of 6.0 and 7.7%, respectively). Dilution of serum or plasma citrate buffer extracts over an eightfold range yielded plots parallel to the standard curve, indicating the absence of enzyme inhibitors or activators in the citrate buffer extracts. The mean oxalate concentrations in serum and plasma—measured in nine and six male volunteers, respectively—were 0.83 ± 0.24 mg/L (SD) and 0.96 ± 0.24 mg/L (SD).

Additional Keyphrases: Kf for phosphate, sulfate · enzyme kinetics · normal values (men)

Because oxalic acid in the presence of oxalate decarboxylase (oxalate carboxy-lyase, EC 4.1.1.2) decomposes to formic acid and CO2 (1), previously reported enzymatic assays for oxalate have depended on measuring the CO2 evolved or the formic acid released. We described elsewhere a radioenzymatic isotope-dilution assay for urinary oxalate (2) involving oxalate decarboxylase, which is relatively simple, accurate, specific, and sensitive and alleviates problems of earlier urine enzymatic methods (3–6). Here we describe a modification of this assay, which enables measurement of oxalate in either serum or plasma. The progress of the reaction is followed by using [14C]oxalic acid and measuring the 14C evolved. Addition of unlabeled substrate under these conditions results in two opposing phenomena. First, the addition of unlabeled substrate to labeled substrate will decrease the specific activity of the substrate, and incorporation of label from substrate into product will be decreased according to sub-stoichiometric isotope dilution (7). Second, in opposition to the first process, at substrate concentrations below about 10 Km production of 14C will be increased because of the greater velocity of the enzyme reaction associated with the increased substrate concentration. The magnitude of these effects is quantitatively predictable from the sub-stoichiometric isotope-dilution principle (7) and Michaelis–Menten (8) kinetics. Combining the isotope-dilution effects and the enzyme-activity effects, Newsholme and Taylor (9) derived an equation for a standard curve:

\[
\frac{C_o}{C_s} = 1 + \frac{S}{(K_m + S)}
\]

where C0 and Cs are the total amount of 14C from [14C]oxalate incorporated into 14C-labeled product with labeled substrate only (C0) or with unlabeled substrate (Cs) also present. S0 and S represent the concentration of labeled and unlabeled substrate, respectively, and Km is the Michaelis constant. A plot of C0/Cs vs S will be linear, with a slope of 1/(Km + S0). C0/Cs will be unity when S is zero. When S0 >> Km, the slope of the line will be 1/S0. This plot is preferable to the linear plot for the standard curve described in our previous urinary assay (2) because the Newsholme-Taylor equation, when rearranged, can yield a value for Km that can be used to check uniformity with each assay:

\[
K_m = \left[ \frac{S}{(C_0/C_s - 1)} \right] - S_0
\]

Material and Methods

Isotope Preparation

Dilute [14C]oxalic acid (sp. act., 40–60 Ci/mol; Amersham Corp., Arlington Heights, IL 60005) in water to 100 mCi/L and store in 25-μL aliquots at −90 °C. Dilute each aliquot to about 40 000 cpm/0.1 mL with citrate buffer (0.1 mol/L, pH 3.0). Make this dilution with each assay. Under the assay conditions some 14C evolves from this solution even when no enzyme is present, ranging from slightly above background to ~100 cpm. Therefore, include blanks with every assay. Refrigerate all solutions when not in use.

Enzyme Preparation

An enzyme unit is defined by each supplier (Worthington Biochemical Corp., Freehold, NJ 07728; Sigma Chemical Co., St. Louis, MO 63178; Aldrich Chemical Co., Milwaukee, WI 53223; U.S. Biochemical Corp., Cleveland, OH 44122) as the amount of enzyme releasing 1 mol of CO2 per minute at 37 °C and pH 3.0. Dissolve the oxalate decarboxylase in 0.1 mol/L citrate buffer, pH 4.5, so that each milliliter contains 2 units of activity, calculated from the information on the vial. To achieve greater uniformity of activity between enzyme batches, dialyze each batch of enzyme against 2 L of 0.1 mol/L
citrate buffer (pH 4.5) for 24 h at 4 °C and freeze 0.1-mL (0.2-unit) aliquots at −90 °C.

To determine what dilution of enzyme to use, set up a series of four "zero" tubes and four tubes containing 4 µg of oxalate standard as described below under Enzyme Assay. Thaw 0.1 mL of enzyme (0.2 units) and dilute with citrate buffer (0.1 mol/L, pH 3.0) to 10 milliliters/0.1 mL, to 7.5 milliliters/0.1 mL, to 5 milliliters/0.1 mL, and to 2.5 milliliters/0.1 mL. Add 0.1 mL of each dilution to the "zero" and 4-µg tubes and incubate as described under Enzyme Assay. Use the dilution of enzyme that evolves between 10,000 and 12,000 counts from the "zero" tube and 1000 to 1200 counts from the 4-µg tube in 8 min for the assay.

**Serum or Plasma Preparation**

Add [14C]oxalic acic (20,000 cpm) as an internal standard to 15 mL of fresh serum or plasma and measure the radioactivity in a 0.1-mL aliquot. Ultrafiltrate the serum or plasma, using Centriflo membrane cones (CF50A or CF25; Amicon, Lexington, MA 02173) in a refrigerated centrifuge at 750 X g for 60 min. Neutralize a 5.0-mL aliquot of the clear ultrafiltrate to pH 7.0 ± 0.1 with 0.1 mol/L HCl. Add 0.5 mL of 20 g/L CaCl2·2H2O followed by 95% ethanol to make the final concentration of alcohol 750 mL/10 mL. Mix gently and allow this solution to stand for at least 3 h (preferably overnight) at room temperature. Centrifuge the sample at 4 °C at 800 X g for 15 min, discard the supernatant fluid, and drain the tube. Dissolve the precipitate in 1 mL of 0.3 mol/L hydrochloric acid and extract with 10 mL of anestheting-grade diethyl ether, saturated with dilute (0.3 mol/L) HCl. Transfer the ether layer to a scintillation vial and evaporate it under nitrogen at room temperature. Repeat the ether extraction and evaporation four more times, placing the extracts in the same vial. At this point the oxalate in the sample is stable when stored at −20 °C. Add 1 mL of sodium citrate buffer (0.1 mol/L, pH 3.0) to the residue and agitate vigorously. Measure the oxalate in this citrate buffer extract by the enzyme assay and, to correct for procedural losses of oxalate, determine the radioactivity of the internal standard in a 0.1-mL aliquot.

**Enzyme Assay**

Assay each sample in triplicate.

Place into 15 X 85 mm glass tubes:
- 0.2 mL of citrate buffer extract of serum or plasma or oxalate standard in 0.1 mol/L citrate buffer (pH 3.0);
- 0.1 mL of ethylenediaminetetraacetate (EDTA), 0.05 mol/L (pH 4.5); and
- 0.1 mL of [14C]oxalate in 0.1 mol/L citrate buffer (pH 3.0) (approximately 40,000 cpm).

Warm the tubes to 31 °C and add 0.1 mL of enzyme in 0.1 mol/L citrate buffer (pH 3.0) to each tube at 30-s intervals. Seal each tube immediately with a rubber cap (cat. no. K-882310; Kontes of Illinois, Evanston, IL 60204) holding a plastic well (cat. no. K-882320, Kontes) containing a strip of Whatman No. 3 filter paper (20 X 10 mm) folded in thirds. Before placing it in the well, saturate each filter-paper strip with 25 µL of CO2 absorber (Oxysorb™CO2; New England Nuclear, Boston, MA 02118) and allow to air dry for 10 to 20 min. Position the well about 2.5 cm below the bottom of the cap (2). Swirl the tubes and incubate them at 31 °C for 5 min in a shaker (120 cycles/min). Using a 1-mL syringe fitted with a 20-gauge 25-40 mm needle, inject 0.2 mL of 100 g/L trichloroacetic acid through the cap. Run standards (0.25-4 µg) and samples in triplicate, along with "no enzyme" controls containing 0.1 mL of buffer instead of enzyme and "zero oxalate" tubes containing 0.2 mL of buffer instead of unlabeled oxalate.

Shake the tubes for 60 min at 31 °C to trap evolved 14C.

Remove the strip and measure its radioactivity in a toluene scintillation cocktail containing, per liter, 5 g of 2,5-diphenyloxazole, 0.1 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene, and 34.5 mL of NCS solubilizer (Amersham). Correct for procedural losses by determining the radioactivity of the [14C] oxalate internal standard in 0.1 mL of the citrate buffer extract of serum or plasma ultrafiltrate.

Using a Model 3330 Tri-Carb Liquid Scintillation Spectrometer (Packard-Instrument Co., Downers Grove, IL 60515), we calculated a 14C-counting efficiency of 84% by determining the cpm of the [14C] oxalate standard (New England Nuclear, lot 897-65) with known dpm in the scintillation fluid. A lesser 14C-counting efficiency will proportionately reduce the absolute sensitivity of the assay.

**Assay Calculations**

Obtain values for the y-axis by making the following calculations: If A = evolved 14C cpm from oxalate standards or unknown extract tubes (containing [14C] oxalate, standard oxalate, or serum or plasma extract, EDTA, and enzyme), and B = evolved 14C cpm from "no enzyme" control tubes (containing [14C]oxalate, EDTA, and buffer), and Z = evolved 14C from "zero oxalate" control tubes (containing [14C]oxalate, EDTA, buffer, and enzyme), then A − B = Cx (corrected cpm for standards or unknown) and Z − B = Cy (corrected cpm for zero unlabeled oxalate). Plot Cx/Cy on the y-axis.

Plot the micrograms of unlabeled oxalate on the x-axis. Calculate the oxalate concentrations of the unknown extracts in micrograms from the standard curve (Figure 1), which satisfies the equation for a straight line: y = mx + b, where y = Cx/Cy, x = micrograms of unlabeled oxalate in assay tube, m = slope of the standard curve, and b = y-intercept = 1.0.

Finally, calculate milligrams of oxalate per liter of serum or plasma = x X R X 1000, where x = µg of oxalate in assay tube, and R (recovery) = (cpm of 0.1 mL of serum or plasma X 50)/(cpm of 0.1 mL of ultrafiltrate citrate extract X 10). The 50 corrects to total volume of serum or plasma and the 10 corrects to total volume of citrate extract.

**Results**

Oxalate was measured after precipitation as calcium oxalate from serum or plasma ultrafiltrates and extraction with ether and citrate buffer. When protein was removed by precipitation with trichloroacetic acid or perchloric acid, we had poor ana-

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**Fig. 1. Standard curve: unlabeled oxalate per assay tube vs Cx/Cy, where Cx is cpm of the 14C evolved with labeled oxalate substrate only, and Cx is the cpm of 14C evolved with unlabeled oxalate substrate also present.**

Contents of reaction mixture are described in Materials and Methods section. Bars represent standard error.
Table 1. Recoveries of Unlabeled Oxalate Added to Serum *

<table>
<thead>
<tr>
<th>Initial mg/L</th>
<th>Added mg/L</th>
<th>Total mg/L</th>
<th>Found (of added) mg/L</th>
</tr>
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<td>3.23</td>
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<td>0.65</td>
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<td>2.60</td>
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<td>2.71</td>
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</tr>
<tr>
<td>2.29</td>
<td>1.00</td>
<td>3.13</td>
<td>0.84</td>
</tr>
<tr>
<td>2.29</td>
<td>2.00</td>
<td>4.42</td>
<td>2.13</td>
</tr>
</tbody>
</table>

Mean, 95.4%; SD, ±12.6%; SE, ±5.7%; CV, 13.3%.

*Mean of triplicate determinations on triplicate precipitations of pooled serum.

Lithyal recoveries of added oxalate. We determined the recovery in ultrafiltrates of oxalate added to serum or plasma by using a range of added radioactive oxalate from 36 μg/L to 4.5 mg/L in two sets of serum samples. One set was allowed to equilibrate for 30 min; a second set stood overnight at 4 °C. Each set was ultrafiltered. Measurements of radioactivity in the ultrafiltrates indicated that the concentrations of [14C]-oxalate in serum and plasma and their respective ultrafiltrates were the same in both sets.

[14C]Oxalate yielded the same cpm values when measured in citrate buffer, serum, plasma, or their respective ultrafiltrates. Recoveries of [14C]oxalate added to serum or plasma as an internal standard were thus used to determine the combined procedural losses ascribable to ultrafiltration, precipitation, and ether extractions. These recoveries ranged from 80 to 90%. After they were corrected by comparison with the [14C]oxalate internal standard, the recoveries of unlabeled oxalate added to serum averaged 95.4 ± 5.7% (SE) (Table 1). A similar recovery was obtained for plasma (data not shown).

The coefficient of variation (CV) for triplicate determinations of triplicate precipitations of pooled serum that had been stored frozen was 6.0%. We observed a 7.7% CV for evaporated ether extracts of serum stored for several weeks at −20 °C.

The oxalate concentrations after diluting the serum citrate extract over an eightfold range are shown in Figure 2. The proportionality between the oxalate concentrations in diluted serum extracts and the oxalate values from the standard curve indicates the absence of either activators or inhibitors of the enzyme in the serum extract (11). This parallelism also indicates that the substance in the extract has the same effect on 14C production as does oxalate, strongly suggesting that the substance measured in the extract by the enzyme is really oxalate. Parallelism between oxalate concentration and serial dilutions of plasma extracts has also been observed (data not shown).

To better define the assay quantitatively, we performed kinetic studies on oxalate decarboxylase (Worthington Corp., lot no. 96A002). Two methods for estimating Km were used: (a) the concentration of radioactive substrate was increased and the amount of 14C evolved was used to measure the progress of the reaction; these data (not shown), plotted according to Lineweaver-Burk (12), gave a Km of 31.3 μmol/L or 3.13 × 10⁻⁵ mol/L. (b) The concentration of unlabeled substrate was increased in the presence of a constant amount of radioactive substrate and the 14C evolved was measured; calculating the Km from the latter data, used to prepare a standard curve (Figure 1) according to the above equations 1 and 2 of Newsholme and Taylor (9), we obtained a value of 31.0 μmol/L. We made the same studies on enzyme preparations from three other commercial sources, to evaluate their uniformity. The Km for the Aldrich enzyme preparation was 35.5 μmol/L, for the Sigma preparation, 80.0 μmol/L, and for the U.S. Biochemical preparation, 24.0 μmol/L.

To assess the effects on the sensitivity of the assay of inhibitors of oxalate decarboxylase known to be present in urine, serum, plasma, and other biological materials, we determined the Ki (13) for phosphate and for sulfate, which we found to be 12 and 7 mmol/L, respectively.

We measured the concentration of oxalate in fresh serum from nine apparently healthy male volunteers who had fasted overnight (Table 2). The mean was 0.83 ± 0.24 mg/L (SD). The concentration of oxalate in the corresponding fresh plasma from six of the volunteers was also measured. The mean oxalate concentration in plasma was 0.96 ± 0.34 mg/L (SD) (Table 2). There was no significant difference between these means, or between the paired measurements of the six individual serum and plasma samples.

Discussion

The sensitivity of the various serum and plasma enzymatic methods varies from 0.05 mg/L by the method of Knowles and Hodgkinson (14) to 8 mg/L by the method of Crawford and Watts (15). The sensitivity of the double-enzyme method (16) for serum is not reported, but the absolute sensitivity of the method for urine is reported to be 2.0 μg (6). We obtained an absolute sensitivity of 0.5 μg.

The reported interassay CV of other methods ranges from 5% in the continuous-flow method (14) to 11.5% in the double-enzyme method (16). In the other assays for serum or plasma oxalate (14–16) there were no reports of intra-assay

Table 2. Oxalate Concentrations of Fasting, Apparenfly Healthy Men

<table>
<thead>
<tr>
<th>Serum mg/L</th>
<th>Plasma mg/L</th>
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<tbody>
<tr>
<td>0.80</td>
<td>0.84</td>
</tr>
<tr>
<td>1.02</td>
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<tr>
<td>1.26</td>
<td>1.41</td>
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<td>0.82</td>
<td>1.17</td>
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<tr>
<td>0.48</td>
<td>0.46</td>
</tr>
<tr>
<td>1.06</td>
<td>0.77</td>
</tr>
<tr>
<td>0.67</td>
<td>0.67</td>
</tr>
<tr>
<td>0.68</td>
<td>0.68</td>
</tr>
<tr>
<td>Mean</td>
<td>0.83</td>
</tr>
<tr>
<td>SD</td>
<td>±0.24</td>
</tr>
<tr>
<td>Range</td>
<td>0.48–1.26</td>
</tr>
</tbody>
</table>

Note: The parallelism between micrograms of unlabeled oxalate in the standard curve and oxalate concentration in increasing volumes of buffer extract of serum ultrafiltrate. Bars represent standard error.
variation. Our interassay and intra-assay CV's were 6.0 and 7.7%, respectively.

The Warburg manometric assay of Crawhall and Watts (15) is the only assay that measures oxalate directly in the plasma. The continuous-flow method of Knowles and Hodgkinson (14) measures oxalate in serum ultrafiltrates. The double-enzyme method of Hatch et al. (16) measures oxalate in citrate buffer extracts of calcium precipitates prepared from serum ultrafiltrates. Our attempts to measure oxalate in serum deproteinized with trichloroacetic acid or perchloric acid yielded low recoveries of added oxalate, which may be the result of oxalate binding to serum proteins. Chambers and Russell (17) report that oxalate binding to serum proteins is significant at acidic pH. We recovered oxalate quantitatively after ultrafiltering serum and plasma without first adjusting the pH to 10.6, as described by Hatch et al. (16). Our best recoveries were obtained with CaCl₂ precipitation of the oxalate in serum or plasma ultrafiltrates. By our method, the percentage recovery of unlabeled oxalate added to serum (95.4 ± 5.7% SE) was essentially the same as with the double-enzyme method (16) (102.9 ± 5.3% SE) and the continuous-flow method (14) (110 ± 1.8% SE).

All the enzymatic assays share the advantage of the specificity of the enzyme. The two methods measure CO₂ by relatively inaccurate methods: Warburg manometry (15) and titration (14). The double-enzyme method makes formate by the absorbance changes resulting from reaction with formate dehydrogenase (EC 1.2.1.2) and NAD⁺, and thus must be corrected for endogenous substances that cause erroneous absorbances. Each of these oxalate assays depends upon complete quantitative conversion of substrate to product. Our assay, which is based on the enzyme-isotope-dilution principle described by Newsholme and Taylor (9), depends upon the progress of conversion of substrate to product and thus is subject to interference by substances that affect the rate of the enzyme reaction. We avoided interfering substances, as demonstrated by the parallelism in Figure 2, by purifying the oxalate by precipitation from ultrafiltrates and extracting it before measurement. Similar results were obtained by the double-enzyme method of Costello et al. (16).

As pointed out by Newsholme and Taylor, the absolute sensitivity of this assay depends upon the Michaelis-Menten constant (Kₘ) of the oxalate decarboxylase. Better to define the utility and sensitivity of the assay, we determined the Kₘ by two methods, as well as the effects upon Kₘ of two common radicals, phosphate and sulfate, which are present in appreciable quantities in most biological materials. The effect of an inhibitor upon Kₘ can be quantitatively estimated from the relation Kₘ (1 + 1/Kᵢ) (11). The only reported Kₘ (1) for oxalate decarboxylase, 2.05 × 10⁻⁵ mol/L, was determined at 37 °C in the presence of phosphate (50 mmol/L), which is a competitive inhibitor of the enzyme. The hitherto unreported Kᵢ (12 mmol/L) for phosphate does not account for the 100-fold discrepancy between the Kᵢ value of 31 mmol/L we observed and the reported value. The effect of phosphate on the Kᵢ in this case could account only for a fivefold change of Kᵢ. The sensitivity of our method (0.5 μg) was close to the theoretical sensitivity of 0.1 Kᵢ (9) if the true Kᵢ, as we observed, is about 3 × 10⁻⁸ mol/L. The Kᵢ for sulfate, 7 mmol/L, is also reported here for the first time.

The mean oxalate concentration in the serum of healthy men has been reported as 1.17 ± 0.20 mg/L (SD) by the continuous-flow method (14) and 1.30 ± 0.42 mg/L (SD) by the double-enzyme method (16). Our mean was 0.83 ± 0.24 mg/L (SD) (Table 2). Our lower value might have resulted because the nine apparently healthy men were fasting. Conversely, the higher value of Knowles and Hodgkinson (14) might have resulted from the gentle boiling of the ultrafiltrate, which may convert other substances to oxalate. Furthermore, the samples were stored at 4 °C until analysis. We (2) and others (3) have shown that oxalate increases upon storage. In the double-enzyme procedure the calcium precipitates are dried at 105 °C for 30 min, then dissolved in citrate buffer at 75 °C for 15 min, which may produce oxalate from other constituents such as calcium ascorbate. Very recently, a radionuclide-dilution assay (18) for plasma oxalate, which yields values comparable to those by an enzymatic assay (5) at plasma concentrations greater than 1.76 mg/L, yielded a value of 0.115 mg/L for normal volunteers.

We believe our method has several advantages. It requires no specially prepared apparatus. It determines oxalate accurately, specifically, and sensitively by measuring radiolabeled product released by a single enzymic reaction. Analytical results may be obtained in two days. Because of the similarity of the two procedures, urine (2) and serum or plasma oxalate concentrations may be determined simultaneously, facilitating clearance determinations.

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References