Enzymic Assay for Oxalate in Unprocessed Urine, as Adapted for a Centrifugal Analyzer

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In this automated modification of the oxalate decarboxylase method, oxalate can be measured (12 per hour) in acidified but otherwise unprocessed urine. Standard curves are linear up to at least 2.5 mmol/L. When 0.50 mmol of oxalate was added per liter to samples of 18 patients' urines, a mean analytical recovery of 98.5% (SD 3.6%) was obtained. Within-series CVs were 3.4 and 1.0%, between-series CVs 7.3 and 2.7% (n = 15) for oxalate concentrations of 0.31 and 0.61 mmol/L. The lower limit of detection is 25 μmol/L. Concentrations measured with this "direct" method correlated well (r = 0.95) with those measured after precipitation with calcium and ethanol and resolubilization in dilute sulfuric acid. For 17 healthy volunteers the mean urinary excretion of oxalate was 0.37 (SD 0.14) mmol/24 h.

Additional Keyphrases: reference interval • enzymic methods • calculous disease • COBAS B12 • formate in urine and serum

Of the various methods currently available for estimating oxalate in urine (1–9), none appears ideal because none allows oxalate to be estimated specifically in unprocessed urine with equipment generally available in hospital laboratories. In most of these methods the sample must first be purified by precipitation with calcium and the precipitate redissolved before analysis (1–3, 5, 7). The gas-chromatographic methods include in addition a derivatization procedure (2, 3). The ion-chromatographic methods may allow urine to be assayed directly (6, 9), but the necessary equipment ordinarily is not available in hospital laboratories. Of the two enzymic methods previously described (4–7) the oxalate oxidase (EC 1.2.3.4) method cannot be used with unprocessed urine because many urines contain substances that strongly inhibit the enzyme (4, 5).

The oxalate decarboxylase (EC 4.1.1.2) method potentially may be used with unprocessed urines, because it appears to be less influenced by inhibitors in urine (6, 7). Despite this, both Costello et al. (6), who used a manual modification of the method, and Yriberri and Posen (7), who used a Technicon AutoAnalyzer, preferred to measure oxalate in extracts of the urines. With untreated urines they did not obtain reliable estimates, mainly because such urines gave variable and high blank values (values obtained in the absence of oxalate decarboxylase). Many of the recently developed automated or semiautomated analyzers allow absorbance or changes in absorbance to be measured very precisely. Their use may therefore considerably reduce the problems concerned with the variable and high blank values. We therefore decided to adapt the decarboxylase method for use in the COBAS B12 centrifugal analyzer, and here we describe how the method may be used to estimate oxalate in unprocessed urine.

Materials and Methods

Samples and Reagents

Sodium oxalate was obtained from Merck, Darmstadt, F.R.G., [14C]oxalate from Amersham, Bucks. HP7 9LL, England, and sodium formate from Fluka AG, Basel, Switzerland. Oxalate decarboxylase purified from Collybia velutipes and β-nicotinamide adenine dinucleotide (NAD+ grade III) were from Sigma Chemical Co., St. Louis, MO 63178. Formate dehydrogenase (EC 1.2.1.2) was from Boehringer, Mannheim, F.R.G.

Urine specimens were collected during 24 h in plastic bottles containing 20 mL of 5 mol/L HCl and were stored at 4 °C (for up to one week) or at −20 °C. Before analysis, each specimen was mixed well and centrifuged (1000 × g, 10 min).

Procedure

Principles of the analysis. The two auxiliary enzymes function at widely different pH optima and are therefore added sequentially. Firstly, oxalate decarboxylase catalyzes the conversion of the oxalate in the urine to formate at a low pH (about 1.5–3.0). By subsequently adding the formate dehydrogenase and the NAD+ in a strong pH 8.0 phosphate buffer, the pH of the mixture is increased to 7.0–7.5 and the formate now present (the sum of that from oxalate and that initially present) reacts with NAD+ to yield bicarbonate and NADH.

In parallel samples run with no oxalate decarboxylase, only the formate initially present gives rise to production of NADH.

Standards. Prepare standards (0.5, 1.0, 1.5 mmol/L) by dissolving sodium oxalate in distilled water that contains, per liter, 200 mmol of HCl and 100 mmol of NaCl. Store them at −20 °C in 500-μL portions. Each day of analysis, thaw a set of standards and assay them.

The oxalate decarboxylase reagent. Prepare a stock solution by dissolving 20 U of the enzyme in 1 mL of a pH 4.0 buffer containing, per liter, 150 mmol of potassium citrate/citric acid and 10 mmol of disodium EDTA, and store it in 80-μL portions at −20 °C. Freshly prepare the COBAS reagent on each day of analysis by diluting this stock solution 1:40 with the above buffer.

The formate dehydrogenase/NAD+ reagent. Prepare a stock solution by dissolving 80 U of the enzyme in 4 mL of a 800 mmol/L potassium phosphate buffer, pH 8.0, and store in 600-μL portions at −20 °C. Freshly prepare the COBAS reagent on each day of analysis by mixing one volume of the stock solution with two volumes of the phosphate buffer to which has been added NAD+ to give a concentration of 22.5 mmol/L.

Analysis. Prepare two reagent trays, one (the enzyme-reagent tray) containing the standards, the oxalate decarboxylase reagent, and the formate dehydrogenase reagent; and the other (the non-enzyme-reagent tray) containing only the citric acid buffer without oxalate decarboxylase.

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In the multi-run mode the Cobas first pipettes all standards and urine samples together with the decarboxylase reagent. The urine samples are then pipetted once more, into the subsequent cuvettes, this time together with the citric acid buffer not containing the decarboxylase. When the single-run and the start buttons are pushed the rest of the analysis is automatically performed. This includes for all cuvettes a 15-min incubation, reading of blank absorbances, addition of the formate dehydrogenase reagent, a second 15-min incubation, and reading of the final absorbances (see Table 1).

In all runs the analyzer also automatically includes a separate cuvette containing only the reagents.

**Calculation of results.** For each cuvette the Cobas automatically calculates the increase in absorbance, corrects for the reagent blank, and estimates the concentration with use of the concurrently analyzed standards. The concentration of pre-formed formate is taken as the result of the analysis in the absence of oxalate decarboxylase. The concentration of oxalate is estimated as the result of the analysis in the presence of oxalate decarboxylase minus the result for the concentration of formate in the sample initially.

**Results**

The Cobas automatically measures both the blank absorbance and the final absorbance for each cuvette and uses these values to calculate the final result. Blank absorbance can be measured at the end of the first incubation, with oxalate decarboxylase, because there is no change in absorbance when oxalate is converted to formate. Blank values so measured ranged, for 20 patients' urines, from 0.04 to 0.31 (mean 0.18).

When the strong phosphate buffer containing the second enzyme, the formate dehydrogenase, subsequently is added, the pH increases from 2 to 7. To check whether this increased the absorbances of urine, due to turbidity, we incubated the 20 patients' urines and also six standards with reagents not containing the auxiliary enzymes. There was an increase in absorbance when the phosphate buffer was added, but it was equal for the urines, the standards, and the reagent blank, and was in the range 0.114 to 0.125. Thus, with the present method an increase in absorbance above that observed with the reagent blank appears to reflect the specific conversion of formate or oxalate or both.

Standard curves based on oxalate were identical to those based on formate and both were linear in the range tested (0–2.5 mmol/L). The curve was also linear for oxalate dissolved in urine instead of the HCl-NaCl mixture, as shown in Figure 1.

For 20 patients' urines, the concentrations of oxalate as measured after precipitation with calcium and ethanol and resolubilization in dilute sulfuric acid correlated well with those measured directly in the untreated urines (Figure 2). This validates the use of the "direct" assay and indicates little interference in the assay from other urinary compounds. Analytical-recovery experiments further indicated little interference. Thus, for the 18 patients' urines we tested, to which we had added 0.50 mmol of oxalate per liter, the recovery was uniformly high (mean 98.5%, range 90.3–104.0%, SD 3.6%).

![Figure 1. Standard curves with oxalate diluted into urine](image1.png)

![Figure 2. Comparison between oxalate concentrations measured directly and following precipitation and resolubilization](image2.png)
The precision of the method was estimated by analyzing on each of several days, in duplicate, two different 24-h specimens. With 15 sets of observations the within-series CVs were calculated to be 3.4% and 1.0% and the between-series CVs 7.3% and 2.7% for oxalate concentrations of 0.31 and 0.61 mmol/L, respectively. The concentrations of formate in these specimens were 0.35 and 0.50 mmol/L. To estimate the lower limit at which oxalate may be accurately measured, we enriched a patient's urine containing 0.28 mmol of oxalate and 0.16 mmol of formate per liter with minor amounts of oxalate (0.025 and 0.050 mmol/L) and analyzed the three specimens repeatedly (n = 20), in sequence. From this we suggest the lower limit of detection to be 0.025 mmol/L, because in all cases the three specimens were correctly identified in order of increasing concentration. Similar figures (0.012 and 0.021 mmol/L) are obtained if the lower limit of detection is estimated as 2 SD of the within-series CVs.

For 17 healthy volunteers the 24-h urinary excretion of oxalate ranged from 0.16 to 0.67 mmol, with a mean of 0.37 mmol and a SD of 0.14 mmol. Uncorrelated, the 24-h formate excretion ranged in the same volunteers from 0.20 to 1.36 mmol (mean 0.60, SD 0.30 mmol).

Discussion

Compared with the other methods for oxalate (1–9) the present method appears to have several advantages, the main one being that oxalate need not be isolated before the analysis. In addition, because the method is automated, as many as 12 urines may be analyzed within 1 h with only moderate amounts of technician time. Reagent costs are also low because the COBAS requires only small volumes of reagent (50–100 µL per analysis). When the reagents are used as described, $18 is the current cost for analysis of 12 urines and three standards.

The present method has been adapted for use with the COBAS BIO, but it also is probably applicable to other types of analyzers. The two auxiliary enzymes used (oxalate decarboxylase and formate dehydrogenase) function at widely different pH and therefore must be added sequentially, each addition followed by a fairly long (here, 15 min) incubation. Any analyzer capable of doing this may potentially be used with the present method. The COBAS, however, has the additional advantage that it automatically detects and corrects for change in the absorbance of the reagent blank, a plausible situation because formate may be present as a contaminant in preparations of NAD+ (10).

Two different commercial preparations of oxalate decarboxylase, isolated from separate sources, are currently available. The decarboxylase we used here, isolated from Collybia velutipes, appears ideal for use with acidified urines because it functions well all the way between pH 1 and 4 (11). However, another type of oxalate decarboxylase, that present in Aspergillus and made commercially available by Boehringer, is not suitable for use with the present method because it functions only at pH nearer neutral (about pH 5).

Although not documented here, the present method, slightly modified, may also allow estimation of formate in serum. Such measurements are of clinical value in patients poisoned with methanol, large quantities of formate being produced when methanol is metabolized (12). Formate appears in fact to be the principal acid that accumulates and probably is chiefly responsible for development of the acidosi so typically seen after methanol poisoning (12).

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References