Assay of Urinary Oxalate: Six Methodologies Compared

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To assess how well results by different methods for urinary oxalate determinations agree with each other in a clinical setting, we compared six different assays: Hodgkinson and Williams (Clin Chim Acta 36:127–132, 1972), enzymatic, modified Hodgkinson and Williams, gas chromatography, ion chromatography, and “high-pressure” liquid chromatography. For the entire group of samples, the mean value by each method agreed relatively closely, although the enzymatic procedure produced a somewhat higher value. All six methods had large coefficients of variation within (8–58%) and between (15–88%) assays. In addition, analytical recovery by most assays was more than 100% of the added oxalate. Analytical recovery of 10 μg of oxalate added per milliliter of urine specimen ranged from 86 to 257%; for 20 μg/mL it was 83 to 320%. Thus for the six methods evaluated, no single method appeared to be superior to the others.

Additional Keyphrases: kidney disease · calculous disease

Calcium oxalate is a major component of more than two-thirds of renal stones in Western populations. Although most investigations have concentrated on factors influencing the urinary excretion of calcium, recent studies have begun to examine the role of increased urinary oxalate excretion (1–4) in the development of renal calculi. Measurement of urinary oxalate in a clinical setting has thus gained importance in studies on renal stone formation.

To date, the measurement of urinary oxalate has been a major problem for clinical biochemists, despite the large number of methods published on this topic. Most of the available methods are tedious, time-consuming, and subject to large errors if care is not exercised in the handling and measurement of samples (5). For the most part, the superiority of one method over others has not been clearly established.

In this collaborative study we have attempted to compare six different methods for the assessment of urinary oxalate and to determine how well results from each method agree with the others. The six methods are the colorimetric method of Hodgkinson and Williams (6), and a modification of this procedure; “high-performance” liquid chromatography (HPLC), gas chromatography, enzymatic digestion, and ion chromatography with conductimetric detection.

Materials and Methods

Sample Preparation

All samples for this collaborative study were obtained from 60 24-h urine specimens brought to the General Clinical Research Center, University of Texas Health Science Center, Dallas, TX, and selected without conscious bias. Specimens were collected without preservative and kept at 4 °C during collection; infected specimens and those with cloudy precipitates were excluded from this study. Aliquots of each urine specimen were prepared for each participating laboratory according to the laboratory’s specifications (see below). Additional samples prepared included a pooled specimen (for determination of the between-run precision), and specimens with added oxalic acid (10 and 20 μg of oxalic acid per milliliter, for assessment of analytical recovery). Each sample was assigned a number. Samples were then frozen and mailed to each laboratory for oxalate analysis, with instructions as to which samples were to be assayed in each run. In general, all samples were assayed in groups of 10, and all values were reported as micrograms of oxalate per milliliter of urine. The data were then sent to the study monitor, where they were subjected to Tukey five-number summary analysis (7) and nonparametric analysis of variance.

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Procedures

Laboratory 1 determined urinary oxalate according to the procedure of Hodgkinson and Williams (6) without further modification. All samples were acidified to pH <2 with concentrated HCl before being frozen and forwarded to laboratory 1. In previous studies in this laboratory overall analytical recovery of oxalate is 86%, as assessed with [14C]oxalic acid.

Laboratory 2 utilized an enzymatic method as previously reported (8). Briefly, oxalate oxidase (EC 1.2.3.4), prepared from a moss species, converts oxalic acid to hydrogen peroxide and carbon dioxide. Hydrogen peroxide is determined enzymatically with horseradish peroxidase (EC 1.11.1.7), by oxidative coupling of 3-methyl-2-benzothiazolinone hydrazide with N,N-dimethylaniline; the resulting indamine dye is determined spectrophotometrically at 595 nm. Substances in urine that interfere with the enzyme's activity are removed by prior adsorption to ion-exchange resin and oxidation with charcoal. Urine specimens were acidified before being forwarded to laboratory 2. Overall recovery of oxalate averages nearly 100%.

Laboratory 3 measured urinary oxalate according to the procedure of Oltthus et al. (9) with slight modification. Two 3-mL washes of 0.125 mol/L and 2.5 mol/L H2SO4 were used, instead of three 2-mL washes stated in the procedure. The oxalate so obtained from ion-exchange chromatography was then reduced to glycolic acid, which forms a colored compound with chromotropic acid. The overall recovery of oxalate by this procedure is 98%.

Laboratory 4 utilized gas chromatography as previously described (10) with further modification. Oxalate is precipitated with calcium sulfate and then converted to dimethyl oxalate, which is measured by gas chromatography. Urine specimens were not acidified before freezing and shipping to this laboratory. Calculated recovery of oxalate, based on recovery of [14C]oxalate, is >98%.

Laboratory 5 determined urinary oxalate by use of ion chromatography as previously described (11). All studies were performed on acidified urine. In this technique, suitably diluted urine is injected into the ion chromatograph (Dionex System 10 Chromatograph; Dionex Corp., Sunnyvale, CA 94086). After the urinary cations pass through the column, the urinary anions are eluted in potassium carbonate buffer. The potassium ions are then removed and the free urinary oxalate is detected by conductivity.

Laboratory 6 used HPLC to measure urinary oxalates. Because this procedure has not been previously published, we present its details here. Acidified urine samples were adjusted to pH 6.0 with concentrated NH4OH solution.

For each oxalate determination, six vigorously stirred 9.50-mL urine samples were pipetted into 16-mL centrifuge tubes. One tube contained the blank, two tubes contained the "test" urine samples, and the remaining three tubes contained urine with standard oxalate additions of 200, 400, and 600 μg.

The oxalate was completely precipitated by adding 0.50 mL of 80 g/L CaCl2 solution to each tube. After sitting for 2 h at room temperature, all tubes were centrifuged at 20 000 rpm (48 200 ×g) for 10 min. Supernates were decanted and the remaining pellets were washed thoroughly with 1.00 mL of pH 6.86 phosphate buffer. The wash solution was separated from the pellets by a second centrifugation at 20 000 rpm for 10 min.

To dissolve the calcium oxalate in the "test" and standard addition samples, 2.00 mL of 0.5 mol/L HCl was added; 2.00 mL of distilled water was added to the blank.

To prolong the life of the chromatographic column, most of the HCl had to be neutralized; thus, to the "test" and standard addition samples was added 0.50 mL of 3 mol/L NH3 solution, and additional NH3 solution was added dropwise until the pH increased to 2.0. Again, an appropriate volume of distilled water was added to the blank.

Suspended solids were removed by a third centrifugation at 20 000 rpm for 10 min. Supernates were decanted into clean test tubes for chromatographic analysis. Overall analytical recovery of oxalate at this point ranged from 82 to 95% as assessed with [14C]oxalate. Then 5.0-μL samples were injected into a "high-pressure" liquid chromatograph (Waters Associates, Milford, MA 01757) equipped with a Waters Corssil II column. The chromatographic solvent was an aqueous pH 2 phosphate buffer (NaH2PO4, 86 mmol/L and H3PO4, 120 mmol/L). At a flow rate of 2 mL/min, symmetrical oxalate peaks appeared by 35 s. Peaks were detected at 211 nm and a detector sensitivity of 0.10 A full scale. Typically, serial injections of each sample were made until acceptable precision was achieved.

The amount of oxalate in each 9.50-mL urine sample (μg/sample) was calculated from the equation

\[ \text{μg/sample} = \frac{\text{μg std. addn.} \times (\text{PHs} - \text{PHb})}{(\text{PHsa} - \text{PHb})} \]

in which PHsa, PHs, and PHb are peak heights of the standard addition urine, the sample or "test" urine, and the blank, respectively. Division of μg/sample by 9.50 mL gave the oxalate concentration in micrograms per milliliter.

Results

Table 1 summarizes the mean, standard deviation (SD), and standard error of the mean (SEM) for each oxalate assay method, based on the 60 samples sent to each laboratory. In addition, the performances of each method were subjected to the Tukey five-number analysis, which provides a measure of the distribution of values about the median for each method so that the distribution of sample values for all the methods can be compared. From the latter analysis, most of

<table>
<thead>
<tr>
<th>Method</th>
<th>Mean</th>
<th>SD</th>
<th>SEM</th>
<th>Minimum</th>
<th>25th</th>
<th>Median</th>
<th>75th</th>
<th>Maximum</th>
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<tbody>
<tr>
<td>Hodgkinson and Williams (60)*</td>
<td>15.8</td>
<td>9.0</td>
<td>1.2</td>
<td>4.700</td>
<td>10.25</td>
<td>14.65</td>
<td>17.83</td>
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<td>30.1</td>
<td>20.5</td>
<td>2.8</td>
<td>2.900</td>
<td>14.90</td>
<td>27.10</td>
<td>38.60</td>
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<td>Modified Hodgkinson and Williams (60)</td>
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<td>12.9</td>
<td>1.7</td>
<td>1.900</td>
<td>11.13</td>
<td>17.35</td>
<td>25.80</td>
<td>68.90</td>
</tr>
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<td>Gas chromatography (50)</td>
<td>16.8</td>
<td>10.7</td>
<td>1.4</td>
<td>6.000</td>
<td>11.00</td>
<td>15.00</td>
<td>24.00</td>
<td>53.00</td>
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<tr>
<td>Conductivity (50)</td>
<td>18.9</td>
<td>10.5</td>
<td>1.4</td>
<td>0.600</td>
<td>6.85</td>
<td>10.00</td>
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<td>HPLC (41)</td>
<td>13.3</td>
<td>11.6</td>
<td>1.8</td>
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</table>

*Numbers in parentheses: no. samples analyzed.
the oxalate assays had 50% of the measured values within a narrow range about the median. The exception was the enzymatic method, in which the median was displaced to the right (higher value) with a somewhat larger 25 to 75th percentile range.

Between-group comparisons for each method were performed by nonparametric analysis because the groups did not have equal variance. This type of data analysis indicated that the mean value for HPLC was significantly lower than the remaining groups, whereas the mean for the enzymatic method was significantly higher. For the remaining methods there was no significant difference among their reported mean oxalate values.

Table 2 summarizes our estimates of intra- and interassay precision.

Accuracy of each oxalate assay was assessed on eight different urine specimens to which 10 or 20 μg of oxalate had been added per milliliter. The results (Table 3) indicate that oxalate accounted for by most of the oxalate assay methods exceeded the actual amount of oxalate added to the specimens. However, each method had a relatively large CV for this determination, making it difficult to compare the relative accuracies of each assay.

Discussion

Although many methods for measuring urinary oxalate have been reported, no study until now has evaluated how results by several assays inter-compares for the same group of urine samples. For the entire group of samples, there was a relatively close agreement between each laboratory's mean value, although the enzymatic method had a somewhat higher median value and range of values than the other methods. Also, when statistical analyses were applied, both the enzymatic and HPLC methods were significantly different from the other four methods (nonparametric analysis). The reasons for these differences are not known. It cannot be due to differences in yield of oxalate after precipitation or separation, because all methods had comparable analytical recoveries of oxalate. Nor can the difference in values observed for the enzymatic method be ascribed to the presence of a reported inhibitor of oxalate oxidase in urine (12), because the observed values in this study were higher than those for the other method. Perhaps some urine specimens might contain an activator of enzyme activity, which is not removed by the ion-exchange resin and subsequent charcoal oxidation. Thus, results by the Hodgkinson and Williams method and its modification, along with gas chromatography and ion chromatography, appeared to agree well with each other. HPLC appeared to underesti-

| Table 2. Intra- and Interassay Variation for Six Urinary Oxalate Assay Methodologies |
|---------------------------------|-----------------|-----------------|-----------------|
| Oxalate conc., μg/mL, mean ± SD (and CV, %) | Intra-assay a | Interassay b |
|-----------------|-----------------|-----------------|-----------------|
| Hodgkinson and Williams | 9.6 ± 1.3 (13.5) | 11.3 ± 2.1 (18.6) | |
| Enzymatic | 37.3 ± 3.0 (8.0) | 17.5 ± 6.0 (34.2) | |
| Modified Hodgkinson and Williams | 15.4 ± 8.9 (57.7) | 16.7 ± 4.9 (29.3) | |
| Gas chromatography | 9.6 ± 2.0 (20.6) | 9.8 ± 1.5 (15.3) | |
| Conductivity | 14.7 ± 3.5 (23.8) | 12.3 ± 3.5 (28.4) | |
| HPLC | 3.6 ± 0.8 (22.3) | 5.3 ± 4.7 (88.6) | |

a Intra-assay variation determined by assessing the same urine specimen four times in the same assay. b Interassay variation determined by assessing the same urine specimen (specimen different from that used for intra-assay variation assessment) in five different assays. *n as in Table 1.

Table 3. Comparison of Accuracy for Six Oxalate Assay Methodologies |

<table>
<thead>
<tr>
<th>Method</th>
<th>Mean oxalate recovery, % (and CV, %) after addition of oxalateacid</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>10 μg/mL</td>
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<tr>
<td>Hodgkinson and Williams</td>
<td>121 (20.4)</td>
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<tr>
<td>Enzymatic</td>
<td>237 (41.7)</td>
</tr>
<tr>
<td>Modified Hodgkinson and Williams</td>
<td>86 (56.9)</td>
</tr>
<tr>
<td>Gas chromatography</td>
<td>140 (17.9)</td>
</tr>
<tr>
<td>Conductivity</td>
<td>159 (48.4)</td>
</tr>
<tr>
<td>HPLC</td>
<td>127 (49.6)</td>
</tr>
</tbody>
</table>

* Each laboratory received five separate urines, with and without 10 μg of added oxalic acid per milliliter. The difference between the two values represented the amount of recovered oxalate for each urine. The same procedure was used for the 20 μg/mL recovery study, except that three urines were used.

mate urinary oxalate concentrations, whereas the enzymatic procedure tended to overestimate the oxalate concentration.

An unexpected finding in this collaborative study was the large CVs observed, most being two to threefold greater than those previously reported for each individual assay. Perhaps this was due, in part, to different personnel performing the assay on different days or problems in establishing a new assay in a laboratory.

Another surprising finding was that five of six methods recovered between 121 and 237% of added oxalate at 10 μg/mL, and three of five recovered 141 to 326% when 20 μg/mL was added. This suggests that some interfering substances in urine were affecting practically all of the methods we evaluated.

We conclude that none of the six oxalate assay methods evaluated here is clearly superior to the others. Obviously, each method has its advantages and drawbacks. Results by the Hodgkinson and Williams method (6) and its reported modification (9) appeared to agree quite well, as would be expected. Both had the best recovery values for 10 μg of added oxalate. However, a potential drawback to these two methods is that the separation of oxalate in these procedures may not always be complete for the modification. The gas chromatographic and ion-conductivity assays also performed well in this study with respect to the mean values for all 60 samples. Despite the large CVs and excessive recovery of oxalate, these methods appear to perform reliably in a clinical setting. The obvious limitations are that the required equipment is expensive and the methods are too time-consuming for routine use. The HPLC has the same drawbacks. Finally, enzymatic degradation would appear to be a sensitive assay system, but expense of the enzyme and the presence of factors in urine that can inhibit or stimulate the enzyme may limit this procedure's usefulness. Thus the choice of which urinary oxalate assay to use will depend on equipment and reagent availability, the number of samples to be assayed, and the projected turn-around time.

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References

Time-Course of Cigarette Smoke Contamination of Clinical Hydrogen Breath-Analysis Tests

Andrea Rosenthal and Noel W. Solomons

The time-course of the contamination of exogenous hydrogen from cigarette smoke on postprandial breath hydrogen concentration was evaluated in 10 subjects, six regular smokers and four occasional smokers. Breath hydrogen values were determined by gas chromatography 10 min, 5 min, and immediately prior to smoking a filter cigarette; during smoking from a sample of exhaled air containing smoke; and 5, 10, and 15 min after extinguishing the cigarette. A three- to 137-fold increase above basal hydrogen concentrations was produced by exhaled cigarette smoke, but most subjects had re-equilibrated to baseline values within 10 to 15 min after the cigarette. If subjects undergoing clinical hydrogen breath tests cannot refrain from smoking during the duration of the test, one should allow an interval of at least 15 min from the end of smoking to the collection of a breath sample.

Additional Keyphrases: variation, source of gas

The hydrogen breath-analysis test has been used in gastrointestinal diagnosis for over a decade (1), its primary application being in evaluation of lactose malabsorption (2-4). The technique is based on the principle that hydrogen is produced in the colon by normal fecal bacteria when ingested carbohydrate escapes complete absorption in the small intestine; a fixed fraction of this colonic hydrogen is absorbed into the bloodstream and excreted by the lungs (5).

Although the original test was based on continuous collection of expired air in a closed system (6), subsequent procedures for breath H₂ tests involved sampling the air at fixed periods after the oral carbohydrate load (2, 3, 7).

Tadesse and Eastwood (8) reported a substantial increase in breath H₂ and CH₄ concentrations when cigarettes were smoked during the course of a breath test. Because the contaminant gases in cigarette smoke are of exogenous origin and poorly soluble in blood, they postulated that the interference caused by smoking would be transient. However, they studied only five subjects. In their report, they provide only mean data, no individual data or any expression of variance around the mean, and no indication of whether the subjects were fasting or in the postprandial state at the time of study.

We encountered a subject with unexpectedly high and erratic values for H₂, unexplained until she was discovered smoking surreptitiously during the course of a lactose absorption test. We then undertook to re-evaluate the issue of cigarette-smoke contamination of expired air in the context of a H₂ breath test.

Materials and Methods

Ten healthy subjects participated in the study. Six were regular smokers, who consumed at least 20 cigarettes per day; the remaining four were occasional smokers. They ranged in age from 20 to 39 years, and none had obvious clinical manifestations of chronic lung disease. One subject was studied in the fasting state; the remainder were studied at various intervals after meals.

Samples of mixed, expired air were collected by having subjects breathe through a low-resistance, one-way Hans Rudolph valve into a 5-L gas-bag. Breath H₂ concentration was measured in a gas chromatograph (Microlyzer Model 12; Quintron Instruments Co., Milwaukee, WI 53215), calibrated with a standard gas mixture containing 100 μL/L of H₂ in N₂ (Scotty Gas II; Supelco Inc, Bellefonte, PA 16823) (9, 10). Samples of air were collected 10 min, 5 min, and immediately after the test filter cigarette was lit. While smoking, subjects exhaled breath containing cigarette smoke directly into the collection bags after each of four or five puffs on the cigarette. The subjects smoked the cigarette...