Factors Affecting Measurement of Urinary Oxalate

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Using a gas-chromatographic method, we examined the effects of phosphate concentration, added calcium chloride, and pH on precipitation of oxalate from urine. All three factors are important, but the pH of precipitation is particularly so, especially in the presence of even normal concentrations of ascorbic acid. At pH 8, increases in measured oxalate ranged from 20% at an ascorbic acid concentration of 1 mmol/L to more than 300% at 15 mmol/L. Ascorbic acid is rapidly converted to oxalate at alkaline pH. We also investigated the stability of both untreated and acidified urine containing ascorbic acid during storage for up to one month at −70, −20, and 4 °C, and room temperature. After one month, untreated collections were stable at −70 °C and acidified collections at −20 and −70 °C. We recommend conditions for assay and storage of urine specimens that are to be assayed for oxalate under which positive interference by ascorbic acid is minimized.

Additional Keyphrases: pH · ascorbate and its precursors · phosphate · calculous disease · sample collection and handling · gas chromatography

For several years, determination of oxalate in urine has been considered important in diagnosing and treating patients with primary hyperoxaluria or oxalate kidney-stone disease (e.g., 1, 2). The numerous methods for measuring oxalate in urine include gas chromatography (3–5), "high-performance" liquid chromatography (6, 7), ion-chromatography (8, 9), enzymic assays (10–14), colorimetry (15, 16), and isocapnophoresis (17, 18). There are also many initial urine "clean-up" procedures, precipitation of oxalate from urine being common to a number of them (3, 10–12, 15–17).

Using a modification of the gas-chromatographic method for urinary oxalate of Farrington and Chalmers (3), we found that the amounts of urinary oxalate precipitated varied widely when we varied urinary phosphate or calcium concentrations or the pH of precipitation.

Moreover, prompted by reports that ascorbic acid interferes with measurement of urinary oxalate in several different methods (7, 12–14, 19–24), we investigated the effect of various concentrations of ascorbic acid on urinary oxalate determination in relation to pH of precipitation, the rate of conversion of ascorbic acid to oxalate at alkaline pH, and the stability of urine stored at various temperatures for up to one month. From these data, we determined and report here conditions for assay of urinary oxalate in which interference by ascorbic acid is minimized. These conditions are applicable to all urinary oxalate methodologies.

Materials and Methods

Materials

To measure urinary oxalate, we modified the gas-chromatographic method (3) as follows. We added no phosphate buffer to the urine, because this interferes with the precipitation of oxalate. Depending on the pH of precipitation, we used the indicators bromcresol green for pH 5 and 6 or bromthymol blue for pH 7, 8, and 9 (both from Merck, Darmstadt, F.R.G.). We adjusted the pH very carefully, with the aid of a pH meter, by adding 1 mmol/L sodium hydroxide solution dropwise, vortex-mixing, and then adding 0.1 mol/L sodium hydroxide to adjust the pH to within 0.1 pH unit of the required value.

In determining the analytical recoveries of oxalate with [U-14C]oxalate, we dissolved the calcium oxalate precipitate in 6 mol/L hydrochloric acid to give a total volume of 2 mL; otherwise, the precipitate was dried directly.

We measured ascorbic acid in urine by "high-performance" liquid chromatography (HPLC), using the method of Wagner et al. (25). We used a Model 334 liquid chromatograph with Model 112 pumps and a Model 185 detector (all from Beckman Instruments Inc., Berkeley, CA 94710).

Samples

Urine specimens were collected either without stabilizer ("plain" collection) or into bottles containing 50 mL of 3 mol/L hydrochloric acid ("acidified" collection). All urine specimens (24-h or untimed) were adjusted to pH <1.6 and well mixed before we took aliquots for oxalate analysis.

Procedures

Effect of calcium chloride concentration and pH of precipitation on precipitation of oxalate and phosphate from urine.

In the case of urine specimens with high phosphate content (>60 mmol/L) we precipitated oxalate by adding 2, 4, 5, 6, 7, or 10 g of calcium chloride per liter and adjusting the pH to 5, 6, 7, or 8. We determined the percentage of oxalate precipitation by using [U-14C]oxalate as described previously (3), except that, owing to the large amount of precipitate, we diluted to a total volume to 3 mL with 6 mol/L hydrochloric acid. Because of the difference in quenching between urine and 6 mol/L hydrochloric acid, we determined the maximum analytical recovery of [U-14C]oxalate by counting the radioactivity of 50-μL aliquots from 3 mL of 6 mol/L hydrochloric acid to which 5 μL of [U-14C]oxalate solution had been added. To measure the amount of phosphate precipitated, we diluted the dissolved precipitate 20-fold and used a continuous-flow procedure (SMA AutoAnalyzer; Technicon Instruments Inc., Tarrytown, NY 10591).

Unless otherwise stated, standard conditions for precipitation of urinary oxalate were: add 2 mL of 5 g/L calcium chloride to 2 mL of urine and adjust the pH to 5, then add 14 mL of ethanol.

Effect of ascorbic acid on measurement of oxalate in urine.

a) pH of precipitation. To 24-h urine specimens collected in acid we added ascorbic acid (Merck) to final concentrations of 0, 1, 5, and 15 mmol/L. Aliquots were assayed for oxalate as described, with the pH for precipitation of oxalate being carefully adjusted to 5, 6, 7, or 8.

b) Duration of alkalization. Aliquots of urine samples supplemented with ascorbic acid as in (a) were alkalized to pH 9 with 1 mol/L sodium hydroxide for 1, 5 and 30 min, after which the samples were adjusted to pH 5 and allowed to precipitate overnight with calcium chloride and ethanol.

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c) Conditions for collection and storage of urine. We stored 5-mL aliquots of urine samples, supplemented with ascorbic acid as in (a), for one month at -70, -20, and 4 °C, and at room temperature (approximately 25 °C). These samples were then assayed for oxalate.

In another study, we obtained 24-h urine collections from two healthy women who had been taking 4 g of ascorbic acid daily for more than three weeks. During the 24-h collection period, each urine sample was halved. One half was transferred to a bottle without stabilizer, the other to a bottle containing 50 mL of 3 mol/L hydrochloric acid. During collection both halves were stored at room temperature. After the collections were completed, the plain urines (pH 5.8) and acidified urines (pH 1.1) were aliquoted and stored at -70, -20, or 4 °C, and at room temperature. Samples were assayed for oxalate immediately and again after storage for 2, 9, 15, and 29 days at the specified temperatures. Before assay, we acidified the plain aliquots (10 mL) by adding 200 μL of concentrated hydrochloric acid.

Results

When we used the original gas-chromatographic method (3) without modification, analytical recoveries of oxalate after precipitation were widely variable and there was a negative correlation between the percentage of oxalate precipitated (as determined with [U-14C]oxalate) and the phosphate concentration in the urine (r = -0.86, n = 22, p < 0.0005) (Figure 1). To improve this precipitation, we investigated the variables involved. Initial studies on factors affecting percentage of oxalate precipitated showed the following: (a) addition of phosphate buffer decreased precipitation, (b) increasing phosphate concentration in the urine decreased precipitation, (c) increasing calcium chloride concentration increased precipitation, and (d) changing the pH of precipitation altered precipitation. More detailed experiments with urines naturally high in phosphate (>60 mmol/L) gave highly variable but reproducible precipitation of oxalate and phosphate, depending on the concentration of added calcium chloride and the pH of precipitation (Figure 2). At pH 7 or 8, more calcium was required to precipitate the oxalate than at pH 6, where recoveries of 90% and greater were obtained even with the lowest concentration (2 g/L) of calcium chloride (Figure 2A). Phosphate showed a consistent trend: increasing calcium chloride concentra-

Fig. 2. Effect of added calcium chloride on precipitation of (A) oxalate and (B) phosphate at various pH

After calcium chloride was added, the pH of a high-phosphate urine was adjusted to the value shown before ethanol was added for overnight precipitation

tion and pH caused more phosphate to precipitate, with 85% of phosphate being precipitated at pH 8 on addition of only 2 g of calcium chloride per liter (Figure 2B).

From our initial work, the pH at which the precipitation step was done also appeared to cause a change in the oxalate measured (after correction for percentage precipitation). A higher pH of precipitation gave a higher value for apparent oxalate. Ascorbic acid in solution is spontaneously converted to oxalate on standing, particularly under alkaline conditions (23, 24, 26, 27). Hence we investigated the effect of ascorbic acid addition on urinary oxalate measurement.

We carried out the precipitation step at pH values of 5, 6, 7, and 8 in the presence of up to 15 mmol/L added ascorbic acid (Figure 3). At pH 5 and 6, the increase in the measured oxalate value was negligible with up to 15 mmol of added ascorbic acid per liter (pH 5 < 10%, pH 6 < 15%). However, when the precipitation was done at pH 7 or 8, the measured oxalate markedly increased. For one

Fig. 3. Urinary oxalate concentration as a function of added ascorbic acid, with oxalate precipitated at various pH
urine, for example, the presence of 15 mmol of ascorbic acid per liter resulted in >200% increase in the oxalate value at pH 7 and >300% increase at pH 8. At pH 8, even 1 mmol of added ascorbic acid per liter produced a 20% increase. Results were similar for other 24-h urine specimens treated in the same manner.

Ascorbic acid is rapidly converted to oxalate at alkaline pH (Figure 4). Exposure to pH 9 for only 1 min resulted in an increase of at least 40% in measured oxalate with addition of only 1 mmol of ascorbic acid per liter. After exposure for 30 min the increase was more than 200%. Conversion was more rapid in the presence of higher concentrations of added ascorbic acid, and partly depended on the urine specimen itself. However, a consistent picture was seen, as shown in Figure 4, with all urine treated as described.

Throughout the ascorbic acid experiments we obtained consistent analytical recoveries of oxalate on using 5 g of added calcium chloride per liter over the pH range examined, with or without added ascorbic acid. Recoveries for these experiments were 96.9% (SD 3.2%, n = 100), based on [U-14C]oxalate.

As our standard conditions for assaying urinary oxalate we chose addition of 2 mL of 5 g/L (45 mmol/L) calcium chloride to 2 mL of urine and precipitation at pH 5 for urine specimens with phosphate concentrations of 40 mmol/L or less. When urinary phosphate was greater, which was infrequent, we added more calcium chloride (2 mL of a 7 g/L solution) to ensure good recoveries of oxalate. Interference by ascorbic acid with oxalate measurement is least at pH 5. Also, at this pH less calcium chloride is required to precipitate oxalate in the presence of high phosphate than at pH 7 or 8; 2 mL of a 5 g/L solution suffices to give >90% recovery for most urine specimens; more is undesirable because more phosphate and other urinary constituents will precipitate. Analytical recovery was 95.8% (SD 3.8%) for 50 different 24-h urine collections under these conditions. During 20 weeks, the between-run CV for two urine controls was 5.4% at an oxalate concentration of 0.23 mmol/L and 4.4% at 0.58 mmol/L (n = 19 each). We made the urine controls by adding potassium oxalate to an acidified urine collection and storing 5-mL aliquots at −20°C.

Figure 5 depicts the stability of acidified urine supplemented with ascorbic acid after storage at the four different temperatures for one month. Oxalate was unchanged when urine was stored at either −70 or −20°C with up to 15 mmol of added ascorbic acid per liter. However, we found increases in measured oxalate in urine stored at 4°C or room temperature. Addition of 15 mmol of ascorbic acid per liter resulted in a 30% increase at 4°C and a 55% increase at room temperature.

Figure 6 illustrates the effect on urinary oxalate measurement of urine collection into plain versus acid containers and storage for one month at different temperatures, for two subjects (A and B) taking oral ascorbic acid. With subject A the acidified collection was stable for urinary oxalate at −70, −20, and 4°C, but increased by over 50% after nine days of storage at room temperature (Figure 6A). The plain collection, on the other hand, was markedly unstable at both 4°C and room temperature, measured oxalate increasing by 30% after only two days at room temperature and for 40% after nine days at 4°C. It was stable for up to one month at −70°C and for 15 days at −20°C. Similar stabilities of plain and acidified urine were obtained for subject B (Figure 6B). These urines had ascorbic acid concentrations of 5 mmol/L for A and 4 mmol/L for B as measured by HPLC (25).

The three immediate endogenous precursors of urinary oxalate in man are ascorbic acid, glycolic acid, and glyoxalic acid (28). We compared the stability of these precursors to alkaline pH by adding them to separate urine samples to give a final concentration of 5 mmol/L and adjusting the

**Fig. 4.** Effect of alkalinization to pH 9 on oxalate measured in the presence of ascorbic acid

Urine supplemented with ascorbic acid was alkalinized for 0 (○), 1 (●), 5 (□), or 30 (○) min, then adjusted to pH 5 for precipitation of oxalate.

**Fig. 5.** Stability, with respect to measured oxalate, of acidified urine supplemented with ascorbic acid after storage for one month at −70°C (○), −20°C (●), 4°C (□), or room temperature (○).

**Fig. 6.** Changes in measured oxalate with storage at various temperatures of plain or acidified 24-h urines collected from two subjects (A and B) who were taking oral ascorbic acid.

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pH to 7 and 10 for precipitation of oxalate overnight. Glycolic acid did not increase measured oxalate; glyoxylic acid gave approximately one-third the increase in apparent urinary oxalate obtained with ascorbic acid (Table 1). The normal value for glyoxylic acid in urine is about 0.03 mmol/L; in children with primary hyperoxaluria it may be 0.15 mmol/L (29). This is too little to cause a significant increase in apparent oxalate in urine under alkaline conditions.

We have shown that ascorbic acid, added to or already present in urine specimens, causes an in vitro increase in oxalate concentration after alkali treatment or storage under certain conditions, when measured by gas-chromatography. Is this increase actually ascribable to production of a compound that has the same retention time as oxalate under the chromatographic conditions employed? To answer this, we measured oxalate by two different methods. An aliquot of urine containing ascorbic acid and another aliquot of the same urine adjusted to pH 10 for an hour then acidified to pH 1 were analyzed for oxalate by gas-chromatography and also by an oxalate oxidase method (13). By gas-chromatography the untreated urine measured 0.37 mmol/L and the alkalinized urine 0.90 mmol/L for oxalate. The corresponding urines gave 0.87 mmol/L and 0.89 mmol/L, respectively, by the oxalate oxidase method. Oxalate oxidase is specific for oxalate (30), so oxalate evidently is in fact being produced by alkali treatment. The untreated urine also gave the higher result for oxalate with the oxalate oxidase method used due to the addition of strong alkali to diluted urine in this method. The authors commented on positive interference by ascorbic acid with their method (13).

Discussion

Our results show that the factors we examined are important.

In the presence of high urinary phosphate, precipitation of oxalate is incomplete unless sufficient calcium has been added, particularly when the precipitation is carried out at pH 7 or 8, at which pH calcium phosphate may be precipitated in preference to calcium oxalate. Analytical recoveries of oxalate by the gas-chromatographic method (3) after precipitation at pH 7 were improved markedly, to 96.0% (SD 3.4%, n = 49) from the original range of 40 to 100%. We did this by omitting the phosphate buffer originally added and increasing the calcium chloride concentration from 2 to 5 g/L (2 mL added to 2 mL of urine). In some other methods (16,29,31-33) for which analytical recoveries of precipitated oxalate are variable, lower amounts of calcium were added than recommended here; recovery might be improved by increasing the calcium for precipitation.

We have shown the importance of pH of precipitation, and of its careful adjustment with dilute alkali. Ascorbic acid in urine can vary considerably, from 0 to 3.4 mmol/day ordinarly up to 16 mmol/day in subjects taking oral ascorbic acid (21). At pH 7 or 8 there is a significant in vitro increase in urinary oxalate in the presence of ascorbic acid over the range possible in human urine (Figure 3), so such pH should not be used for precipitation of oxalate. Ascorbic acid interfered least at pH 5, with good analytical recoveries of oxalate of 95.8% (calcium chloride = 5 g/L). This obviates the need for use of isotope-dilution correction procedures for incomplete precipitation. The rapid rate of conversion of ascorbic acid to oxalate on alkalization of urine to pH 9 (see Figure 4) indicates that any analytical method for oxalate involving addition of alkali to urine may be subject to error unless the pH is kept below 6 and dilute alkali is added slowly with constant mixing. In many methods strong alkali is used (e.g., 5 mol/L NaOH or even NaOH pellets) to adjust the pH of urine for precipitation. This would certainly result in localized regions of high pH in the urine, facilitating conversion of any ascorbic acid present to oxalate.

When establishing a new or modified procedure for urinary oxalate, many workers have used the colorimetric method of Hodgkinson and Williams (15) as the method of comparison (e.g., 3,4,7,8,13,14). This method is not a good choice for comparison because it is subject to error in addition to those already described (15). In this method, oxalate is precipitated from urine at pH 7, which would, as shown by our studies, result in a substantial increase in measured oxalate for urine samples containing more than 1 mmol of ascorbic acid per liter (see Figure 3).

The stability of urine with respect to measured oxalate is also affected by the presence of ascorbic acid, particularly with storage at 4 °C or room temperature, depending on the urinary ascorbic acid concentration and the type of collection (see Figures 5 and 6). Previous workers have commented on the increase in oxalate in urine on storage, even at −15 °C or with added hydrochloric acid (34). Our studies show that urine specimens should be acidified within two days of collection if stored at 4 °C and immediately after collection if stored at room temperature. We also recommend that urine samples be stored frozen (−20 or −70 °C) after acidification. Under these conditions any increase in measured oxalate from ascorbic acid during storage is minimized.

Some ascorbic acid in acidified urine does form oxalate on storage at 4 °C or room temperature, but we find that a much larger proportion forms another, as-yet-unidentified compound, which does not produce oxalate on alkalization of urine. We are currently studying this problem further.

Many methods have been described for assay of urinary oxalate. There is no clear-cut choice for clinical laboratories. A recent study (31) compared six commonly used methods. The CVs for all six methods were large. None was demonstrably superior. Perhaps poor performance of urinary oxalate methodologies in general may be explained by considering the factors we have examined.

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References


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<th>Table 1. Stability of the Three Immediate Precursors of Oxalate in Alkaline Urine with Respect to Urinary Oxalate Measurement</th>
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<tr>
<td><strong>Compound added to urine, 5 mmol/L</strong></td>
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<tr>
<td>None</td>
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<tr>
<td>Ascorbic acid</td>
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<td>Glycolic acid</td>
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<td>Glyoxylic acid</td>
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<td>*pH at which oxalate was precipitated.</td>
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<td>aExtra peak on gas chromatogram, not normally found.</td>
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