Stability of Ascorbate in Urine: Relevance to Analyses for Ascorbate and Oxalate

A. H. Chalmers, D. M. Cowley, and B. C. McWhinney

Ascorbate is unstable in urine at room temperature at pH values ranging from 1 to 12. At pH 7 and above, oxalate is generated in amounts directly proportional to the ascorbate concentration. In 12 different urines, adjusted to pH 12 and incubated for 20 h at room temperature, there was a significant correlation between the amount of oxalate formed and the initial ascorbate concentration (r = 0.97, p < 0.01). The mean (± SD) concentration of oxalate (1.32 ± 0.70 mmol/L) formed during this period approximated the initial ascorbate concentration (1.57 ± 1.09 mmol/L). Disodium EDTA, 10 mmol/L final concentration, stabilizes ascorbate in urine and inhibits its conversion to oxalate at pH values of 4.4 to 7.0 during a 24-h period. We therefore propose that urine specimens for ascorbate and oxalate analyses be collected with disodium EDTA present such as to give about this final concentration.

Additional Keyphrases: EDTA and other compounds as stabilizers
- urinary calculous disease

Oxalate in urine is a major factor in the formation of urinary oxalate calculi (1), making accurate measurement of oxalate in the urine of stone formers important in their clinical management. Ascorbate contributes about 40% of the oxalate in human urine and thus is considered a major precursor of oxalate (2, 3). These are conflicting views as to whether a high intake of ascorbate significantly affects urinary excretion (4–6). Part of this confusion may be explained by the alkaline conditions used in some assays for oxalate in urine, which would result in substantial conversion of ascorbate to oxalate (7, 8).

Recommendations vary as to conditions for collecting 24-h urine specimens for ascorbate analyses. Some collect specimens into (a) metaphosphoric acid at concentrations of 5 to 50 g/L (9–11), (b) 20 mL of concentrated hydrochloric acid and 2 g of disodium EDTA (4), or (c) oxalic acid, 5 g/L (11). Many reports (5, 12–15) mention no ascorbate stabilizers; presumably, those urines were collected without any preservatives. Analyzing each voiding during a 24-h collection for ascorbate immediately after collection or, alternatively, freezing each specimen until assayed, is considered impractical (16). As part of a study on ascorbate metabolism in renal stone formers, we investigated the stability of ascorbate and its conversion to oxalate at different urinary pH values. We also attempted to stabilize ascorbate in urine and inhibit its conversion to oxalate. Here we report the results of this study.

Materials and Methods

Materials. Ascorbic acid, glyoxylic acid, butylated hydroxyanisole (BHA), and all carbohydrates studied were purchased from Sigma Chemical Co., St., Louis, MO. Hydrazine sulfate, sodium thimerosal, boric acid, metaphosphoric acid, and disodium EDTA were from BDH, Sydney, Australia. [U-14C]Oxalate was from Amersham, Sydney, Australia.

Just before assay we prepared aqueous solutions of ascorbic acid at 20-fold the desired concentration in urine. Inhibitors were freshly prepared as neutral aqueous solutions, at 10-fold the final assay-mixture concentrations, except BHA, which was in ethanolic solution.

Analytical methods. We measured urinary oxalate by rate analysis with a specific enzyme method (17). Urinary ascorbate was estimated by a modified 2,4-dinitrophenyhydrazine method (18) in which ascorbate is oxidized with charcoal and Fe3+ to form dehydroascorbate, which is then measured as its colored hydrazone after reaction with 2,4-dinitrophenyhydrazine.

Urinary studies. All urine specimens were collected and maintained at room temperature (20–26°C).

To 20 mL of urine, with or without inhibitors, we added either orthophosphoric acid or NaOH (2 mmol/L solutions) to adjust to the required pH and then diluted to 25 mL with water. As the first step in analyses for oxalate, to 1-mL aliquots of these urines we added 14 mL of ethanol to precipitate the calcium oxalate (17). The first step in ascorbate analyses was to add acidified oxidizing solution (18) to each sample.

In the time-course studies, the time reported is the interval during which ascorbate is present in the urine adjusted to different pH values and does not include the overnight precipitation of calcium oxalate from the urine with ethanol. Because calcium oxalate is precipitated inefficiently in acidified (pH ≤ 2.0) or alkalinized urines (pH ≥ 10), we first adjusted these urines to pHs between 3 and 5 with HCl or NaOH (0.2 mol/L), with methyl red as the indicator, immediately before the precipitation step (17). All other urines were precipitated with ethanol at the pH values to which they had been adjusted. The analyses for ascorbate did not require pH adjustments, because of the high concentration of trichloroacetic acid used (18).

Statistics. To compare results of the two procedures, we used the products moment correlation and Student’s paired t-test (19).

Results

Conversion of ascorbate to oxalate in urine. Figure 1 depicts the short-term stability of ascorbate in urine at
The was a 50-mm analytical bate, to during metaphosphoric ascrobate, and inhibit respectivly. The urine was supplemeted with ascorbate to give a 1 mmol/L final concentration (exclusive of ascorbate initially present) different pH values. Ascorbate appears to be least stable at pH 9.0. The oxalate generated at the various pH values studied was substantial, particularly at pH values = 7.0 (Figure 1).

Increasing the ascorbate concentration at pH 1.5 had no effect on the oxalate concentration in urine. At pH 7.0, however, the oxalate concentration in urines containing 0.26, 2.25, and 5.25 mmol of ascorbate per liter increased, respectively, by 0.015, 0.035, and 0.10 mmol/L by 1 h and 0.055, 0.14, and 0.20 mmol/L by 24 h.

Inhibitor studies. The inhibitors we tested were antioxidants or binders of metal ions, which were expected to inhibit the oxidation of ascorbate (20). Hydrazine, sodium borate, and disodium EDTA (10 mmol each per liter), metaphosphoric acid (18 g/L), and BHA (1.2 mmol/L) were tested for their effect on the conversion of ascorbate to oxalate during 24 h. We adjusted the urines, with and without inhibitors, to pH 1.5, 7.0, and 9.0, then added ascorbate to give a final concentration of 5 mmol/L. Untimed urine specimens were supplemented with metaphosphoric acid immediately after collection (urinary pH values ranged from 1.74 to 2.54). Borate, BHA, and hydrazine did not stabilize ascorbate at these pHs, nor did they completely inhibit the generation of oxalate from its precursors at pH 7.0 and 9.0 (results not shown). Urines stabilized with metaphosphoric acid lost an average of 8% of their ascorbate during 24 h (n = 5 urines). However, oxidation of ascorbate to dehydroascorbate, a step used in the analysis for ascorbate, was inhibited by metaphosphoric acid, decreasing the analytical recovery of dehydroascorbate to 40% even after a 50-min incubation in a highly oxidizing environment (18). The oxalate in urine collected over metaphosphoric acid was stable. Also, in urine with added disodium EDTA, ascorbate was stabilized and oxalate generation was inhibited at pH 7.0 (Figure 2).

We next studied the effect of disodium EDTA, 10 mmol/L final concentration, on the pH values within the normal range for urine. For periods longer than one day ascorbate was more stable in disodium EDTA at the lower pH values (4.5 and 5.8 vs 7.5). Urine oxalate concentrations did not change over four days at pH 4.5, even in the absence of disodium EDTA. At pH 7.5 oxalate concentration had increased by 45% by day 2 and by 500–700% by day 4 as compared with day 0, whether or not disodium EDTA was present. We did not measure oxalate in the pH 5.8 urine. The baseline oxalate value was 0.11 mmol/L.

Next we studied the effect of disodium EDTA on the stability of ascorbate and oxalate in 10 different urine specimens during 24 h. Untimed 25- to 50-mL specimens of urine were collected from healthy laboratory volunteers into disodium EDTA such that the final concentration was about 10 mmol/L. The pHs of these urines ranged from 4.40 to 6.32, 0.73 (± 0.23 SD) pH unit less than before the disodium EDTA was added. The ascorbate concentration in the urines ranged from 0.09 to 1.25 mmol/L and oxalate from 0.06 to 0.48 mmol/L. Ascorbate and oxalate concentrations were relatively unchanged 24 h later, averaging 96.8% (SD 11.0%) and 101.8% (SD 2.8%), respectively, of their original concentrations. Disodium EDTA also prevented oxalate precipitation in frozen urines. The analytical recovery of [U-14C]oxalate from urines stored frozen overnight and thawed was 105.2% (SD 2.1%). The yield of [U-14C]oxalate after precipitation with ethanol from urines containing disodium EDTA was 83.3% (SD 13.0%), which compared favorably with results for urines collected into acid (17).

Alkali-derived oxalate and ascorbate. Urines adjusted to pH 12 with a 5 mol/L solution of sodium hydroxide were analyzed for oxalate before and 5, 20, and 48 h after alkalinization. We determined the oxalate formed in the specimens as the difference between the value obtained after alkali treatment and the oxalate concentration of non-alkalinized urine. We also measured ascorbate concentrations in the urine before and 5 h after alkali treatment.
The correlation between the alkali-derived oxalate and the ascorbate concentrations after 20 h of incubation was significant ($r = 0.97; p < 0.01$), the mean (± SD) alkali-derived oxalate (1.32 ± 0.70 mmol/L) being almost stoichiometrically related to the ascorbate (1.57 ± 1.09 mmol/L). For the 5- and 48-h incubations the alkali-derived oxalate concentrations were lower: 0.87 ± 0.67 and 1.08 ± 0.49 mmol/L, respectively. We did not detect ascorbate in all urines after the 5-h incubation.

We tested fructose, glucose, sucrose, lactulose, arabinose, glyoxylate, maltose, trehalose, and galactose at 5 mmol/L as possible oxalate precursors at pH values of 2, 7 and 11. Oxalate was not generated from any of them over a 24-h period at room temperature. It is likely, therefore, from the stoichiometry of the conversion of ascorbate to oxalate in urine at pH 12, that the principal alkali-labile oxalate precursors in urine are ascorbate and some of its metabolites (20).

**Discussion**

Our results indicate that ascorbate is unstable at various pHs of urine, being most stable at about pH 4.5–5.0. At pH 7.0 there is a considerable conversion of ascorbate to oxalate, and this is important because most methods for oxalate involve an overnight precipitation of calcium oxalate at a pH value near 7.0 (21).

Disodium EDTA stabilizes ascorbate, probably by decreasing the pH of urine and also by binding the metal ions in urine, thereby inhibiting the formation of dehydroascorbate, an intermediate in the conversion of ascorbate to oxalate (16, 20). The results also confirm that oxalates are stable if urine is collected into acid and the calcium oxalate is precipitated at a pH between 3 and 5 (17).

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Guided by our results, we recommend that 24-h urine specimens for ascorbate and oxalate analyses be collected with disodium EDTA (20–25 mmol) and sodium thimerosal (0.2–0.6 mmol) in the collection vessel. The latter compound inhibits bacterial growth and does not interfere with assays for ascorbate and oxalate. Collection of urine into disodium EDTA has an additional advantage in that no further adjustment of urine pH is required for the oxalate analyses. We have recently adopted the method of Urdal (22) for measurement of urinary oxalate. This method does not require precipitation of calcium oxalate with alcohol, and in our experience it works satisfactorily with urines collected into disodium EDTA/thimerosal. For this method, however, accurate results are obtained by preparing the oxalate standards in disodium EDTA (10 mmol/L) instead of the suggested acid/salt mixture (22).

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**References**