Collection and Handling of 24-Hour Urine Specimens for Measurement of Analytes Related to Renal Calculi

Ronald H. Ng, Mani Menon, and Jack H. Ladenson

Analysis for calcium, magnesium, phosphorus, oxalate, uric acid, and creatinine in 24-h urine collections is often needed for the differential diagnosis of patients with renal calculi. Considerable attention has been given to improving the methods of analysis, but improper sample collection and processing can cause significant errors for calcium and oxalate in urine samples not treated with acid and for uric acid in urine samples not treated with base. The errors are related to the concentration of the analyte, the interval the sample is stored before analysis, and the original pH of the urine sample. We describe here a system of sequential acidification (to pH 1.5) and alkalization (to pH 9) of 24-h urine samples, followed by heating at 56°C for 10 min. This procedure allows accurate analysis for all the above analytes in the same 24-h collection of urine. We validated the sample-treatment protocol for 80 24-h urine collections.

Additional Keyphrases: calcium • oxalate • uric acid • creatinine • magnesium • urine analysis • sample handling • variation, source of

The diagnostic evaluation of patients with calcium oxalate renal calculi often requires measurement of calcium, magnesium, phosphorus, uric acid, and (or) oxalate in a 24-h collection of urine (1). Knowledge of the urinary excretion of calcium, oxalate, and uric acid can be particularly important in these patients because calcium excretion is high in about 30% and excretion of uric acid is high in about 25%, and treatment may be predicated on the biochemical values (2–6).

The solubility properties of calcium, oxalate, and uric acid are quite different. Calcium and oxalate are soluble in acid (7), but uric acid is soluble in base (8). Some clinical investigators have considered these differences in solubilities when collecting samples (3), but it is not clear whether others have done so (6, 9–12). Likewise, major reference laboratories do not seem to consider the differences in solubility when recommending collection conditions. Of four such laboratories whose current catalogs we reviewed, none recommended the collection of urine in base when uric acid was to be measured, four recommended acid for collecting calcium samples, but only three recommended acid when oxalate is to be measured.

Some investigators (2) recommend collecting four consecutive 24-h urine samples, the first two for calcium and oxalate and the last two for uric acid. This protocol has a high potential for improper collections and is inconvenient to the patient, because such samples should be obtained while the patients are on their usual diet and daily regime, i.e., as outpatients (1, 2).

Because of the many questions we received from clinicians about the collection conditions for these analytes, we investigated the influence of pH on the accuracy of analyses for calcium, oxalate, uric acid, magnesium, phosphate, and creatinine in urine. We further vigorously evaluated a method of changing the pH of the sample when it reaches the laboratory, as mentioned by others (13, 14). Our data indicate that an inappropriate pH can invalidate results for some of these analytes, whereas a system of changing the pH plus heating can ensure proper results from a single urine specimen.

Materials and Methods

Analytical Methods

Calcium and magnesium were measured by atomic absorption spectroscopy (Jarrell-Ash atomic absorption spectrophotometer, Model 580; Fisher Scientific Co., Waltham, MA 02254). Phosphorus was measured by an equilibrium method involving molybdate, and uric acid by a kinetic method involving uricase, both in a discrete analyzer (aco; Du Pont Co., Clinical Systems Division, Wilmington, DE 19898). Oxalate was measured by ion chromatography with a Dionex 10 chromatograph (Dionex Corp., Sunnyvale, CA) (15, 16). To measure creatinine we utilized a picric acid kinetic procedure and a centrifugal analyzer (CentrifilChem System 400 Analyzer; Baker Instruments, Bethlehem, PA 18001). The interassay CVs were <3% for all assays except oxalate, for which the CV was <5%. Urinary pH was measured either with a combination pH electrode or by colorimetric pH strips scored at 0.2 pH unit gradations (Pehanon; Gallard–Schlesinger Chemical Corp., Carle Place, NY 11514).

Stability of Calcium, Oxalate, and Uric Acid

The stability of these analytes was assessed by use of untimed urine specimens from normal men or from patients with renal stones. Samples were divided into aliquots and the pH adjusted with concentrated HCl or NaOH without delay, either before or after the analyte being studied was added. We stored these samples for various periods before assay. In some cases, we then changed the pH of the samples, heated them, and remeasured the analyte.

Assessment of Treatment of 24-h Urine Collections

Eighty 24-h urine samples were collected without preservative at room temperature from patients at Barnes Hospital or Clinics for whom calcium, magnesium, phosphorus, uric acid, and (or) oxalate analysis had been requested. The laboratory processed the urine within 24 h of receipt. Samples that could not be processed within 2 h were stored.
at 4 °C. We used the following protocol for processing the urine samples:

1. Use a magnetic stirring bar in the 24-h urine specimen to mix the urine continuously.
2. Remove two 10-mL aliquots (untreated sample).
3. Adjust the pH of the remaining urine sample to pH 1.5–2.5 with HCl, 12 mmol/L; remove two 10-mL aliquots and heat to 56 °C for 10 min (acidified sample).
4. Adjust the pH of the remaining urine sample to pH 9–10 with NaOH, 500 g/L; remove two 10-mL aliquots and heat to 56 °C for 10 min (alkalinized sample).
5. Measure the volume of the remaining urine and add 60 mL to the result to obtain the total urine volume.4

All samples (untreated, acidified, alkalinized) were centrifuged at 1000 x g for 10 min, then analyzed promptly or stored at 4 °C. We used acidified and untreated samples for determination of calcium, magnesium, phosphorus, oxalate, and creatinine, and alkalinized and untreated samples for determinations of uric acid and creatinine. To evaluate the significance of differences in values obtained with the various treatment regimes, we used paired-t analysis, after omitting outliers by sequentially applying the internally “studentized” (sic) extreme deviation from the mean (X̄ ± SD) at the 99% level (17).

**Results**

**Stability**

*Uric acid.* When uric acid (330 or 630 mg/L) was added to a urine sample stored at pH 5, the values decreased after storage for 24 h (by 260 and 550 mg/L, respectively). Even after 11 days of storage, alkalinization and heating restored the uric acid concentration to its original value, but neither heating alone nor alkalinization alone sufficed to fully redissolve the uric acid. Results were similar for six freshly collected morning urine samples stored for 24 h (Table 1).

*Calcium.* For 19 fresh urine samples from normal men, we found no significant differences ascribable to the sample-treatment regimes, except that samples at pH 9 had values significantly (p <0.005) lower than untreated samples or samples at pH 1.5 (Table 2). However, the number of outliers found when we compared the different treatments suggests that large differences could occur in some samples if they were not either collected at pH 1.5 or adjusted to pH 1.5 with heating before analysis.

*Oxalate.* Analysis of a urine sample containing 30.2 mg of oxalate per liter indicated that, after adjustment of the pH to 2.5 and 1.5, the oxalate values increased to 13% and 90% more than the original sample. During the next three days the pH 2.5 and 1.5 samples, stored at 4 °C, gave the same oxalate value while the untreated sample gave progressively lower values (25.5 mg/L after 24 h; 19.9 mg/L after 48 h). Greater acidification did not further increase the oxalate.

When we acidified three random samples of urine to pH 1, the measured oxalate concentrations increased in all three. Adjusting the pH back to the original value reversed this increase, and the oxalate values were similar to those measured in the original untreated sample. This suggests a pH-reversible dissolution of oxalate salts. However, alkalinization of urine to pH 10 also increased urinary oxalate by 42%, the extent of the increase depending on the duration of alkalinization. Moreover, even after the urine was reacidified, the value for oxalate was 45% higher than that of the original sample treated only with acid. This suggests

4 pH adjustment required 5–10 mL of acid or base, a volume considered small enough to ignore in calculating the total urine volume.

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**Table 1. Uric Acid Measured in Six Fresh Urine Specimens after 24 h of Storage at Various pH Values**

<table>
<thead>
<tr>
<th>Uric acid concn, mg/L</th>
<th>Assayed without delay</th>
<th>Assayed after 24 h</th>
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<tbody>
<tr>
<td>pH 5</td>
<td>pH 9</td>
<td>pH 5 treated*</td>
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<tr>
<td>380</td>
<td>410</td>
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<td>740</td>
<td>770</td>
<td>760</td>
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</tbody>
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* Untreated
* Adjusted to pH 9 and heated at 56 °C for 10 min.

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**Table 2. Calcium Measured in 19 Fresh Urine Specimens, Assayed Promptly and after 24 h of Storage**

<table>
<thead>
<tr>
<th>pH 1.5</th>
<th>Untreated*</th>
<th>pH 9</th>
<th>Untreated</th>
<th>Untreated, after acidification and heating*</th>
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<td>262</td>
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</table>

* pH 6–7; not significantly different from values at pH 1.5 after elimination of one sample with value 50 mg/L lower than that at pH 1.5.
* Significant differences between untreated samples (p < 0.005).
* Not significantly different from samples at pH 1.5 after elimination of one sample with value 106 mg/L lower than at pH 1.5.
* Not significantly different from untreated after elimination of three outliers with values 92, 58, and 56 mg/L higher than the untreated samples.
* Not analyzed.

that alkalinization of urine causes an irreversible de novo generation of oxalic acid from precursors in the urine. (We find that alkalinization of parabanic acid or oxaluric acid also results in the formation of oxalate.)

**Assessment of Laboratory Treatment of 24-h Urine Specimens**

Having found that acidification or alkalinization with heat could redissolve calcium or uric acid in urine and also allow samples valid storage for at least 24 h, we assessed the influence of our specimen-processing procedure for 24-h urine collections from patients.

For *uric acid*, most samples gave similar values for the untreated and alkalinized aliquots assayed after one day of storage. Three samples had a difference (alkalinized minus untreated) of −80 to −41 mg/L; 42 a difference of −50 to −1; and 31 a difference of 0 to 40. However, we identified four samples as outliers, their differences in uric acid concentra-
tion between the untreated and alkalinized samples being 230, 420, 750, and 770 mg/L.

The mean value for uric acid in the alkalinized samples was 317 mg/L for the 76 samples that showed little difference in values between the untreated and alkalinized samples, a value significantly lower (p < 0.005) than the mean value of 881 mg/L for the four discrepant samples.

The 24-h excretion of uric acid for these patients was within the normal reference interval according to the values for the untreated urine but highly supranormal according to the values for the alkalinized samples. The mean difference between the two treatment groups, after elimination of the outliers, was −5.4 mg/L (p < 0.005). The uric acid values for the untreated samples decreased with time of storage but were unaltered in the alkalinized samples (Figure 1). Thus the degree of underestimation of uric acid in urine samples not treated with alkali depends on the time the sample has been stored before assay and also on the concentration of uric acid in the original sample.

For calcium, three of 78 samples analyzed within 24 h of collection were identified as outliers, the differences between the acidified and untreated samples being +72, −20, and −20 mg/L. As for the remainder of the samples, 22 had differences of −10 to −1 mg/L, 51 had differences of 0 to 10 mg/L, and two had differences of 11 to 20 mg/L. If the three outliers were omitted the mean differences between the two treatment groups was not significant. For 24 samples assayed after seven days of storage, six were identified as outliers with differences between the acidified and untreated samples of 146, 116, 86, 64, 49, and 37 mg/L. For the remainder of the samples, four had differences between −4 and −1 mg/L, 11 had differences between 0 and 10 mg/L, and three had differences between 11 and 18 mg/L. After omission of the six outliers, the mean difference between the two treatment groups was 7.4 mg/L (p < 0.005). The mean original value for calcium (137 mg/L) for the 18 samples that showed little difference in values between the untreated and acidified samples after seven days of storage was significantly lower (p < 0.05) than the mean original value of 200 mg/L for the samples that showed large discrepancies.

Like uric acid, calcium values decreased with time in untreated samples but not in acidified samples (Figure 2).

For magnesium, we considered five of 77 samples to be outliers with differences of 1.4, 1.2, 0.9, −0.5, and −0.4 mmol/L between acidified and untreated samples assayed within 24 h of storage. If the outliers were excluded, the mean difference in values between the two groups of samples was not significant. The distribution of the values (acidified minus untreated) after elimination of the outliers was as follows: four samples had differences between −0.25 and −0.15 mmol/L, 17 samples had differences between 0.1 and 0.05 mmol/L, 48 samples had differences between 0 and 0.1 mmol/L, and three samples had differences between 0.2 and 0.3 mmol/L. The mean original acidified value for the five outliers was 3.54 mmol/L, significantly (p < 0.02) higher than the mean value for the remainder of the samples (2.48 mmol/L). For inorganic phosphorus, two of 78 samples were identified as outliers, the acidified samples minus the untreated samples differing by 104 and −87 mg/L when assayed within 24 h. If the outliers were excluded, the values by the two treatment regimes were not significantly different. The distribution of the differences between the acidified and untreated samples after omission of the outliers was as follows: two samples between −80 and −71 mg/L, eight samples between −30 and −11 mg/L, 48 samples between −10 and +10 mg/L, 10 samples between 11 and 30 mg/L, six samples between 31 and 50 mg/L, and two samples between 61 and 90 mg/L. There was no significant difference in mean values for acidified samples between the outliers and non-outliers. For creatinine, one sample differed by 100 mg/L between untreated and acidified samples; the other 55 samples showed a significant difference (5.8, SD 21.1 mg/L, p < 0.05) between treatments (Figure 3A). The outlying sample had a value of 210 mg/L for the untreated aliquot as compared with a mean value of 72 mg/L for the remainder of the samples. When we alkalinized the samples, one had a difference between untreated and alkalinized of 310 mg/L and the others differed by a mean of 9.6 (SD 20.9) mg/L, p < 0.02 (Figure 3B). The value for the untreated aliquot of
Discussion

Our results confirm the predictions, based on solubility properties, that uric acid can precipitate at low pH values or high concentrations and that calcium and oxalate can precipitate at high pH values or high concentrations. The calcium can essentially be fully redisolved by acidifying and heating the urine sample and the uric acid by alkalinizing and heating, even after storage of the specimen, as has been suggested anecdotally by others (13, 14). For oxalate, we suggest that the specimen be acidified on the same day the urine is collected, to avoid the increases in oxalate that might occur in alkaline urine.

The incidence of clinically significant errors in the assessment of calcium or uric acid excretion from urine collected without preservative depended on how long the samples were stored before analysis; moreover, the samples that tended to give the greatest errors were those with higher concentrations of analyte. These findings are consistent with the known solubility properties of these analytes and suggest that the incidence of erroneous values would be greater in patients with renal calculi who have high urinary concentrations of calcium, oxalate, and (or) uric acid (19).

The protocol we used—sequential acidification and alkalinization with heating—allows accurate determination of calcium, oxalate, and uric acid in the same specimen of urine. An alternative procedure of removing separate aliquots for the addition of acid or base presented problems in obtaining a homogeneous sample, owing to settling of precipitates. Alkalinization of the urine to pH 11 instead of 10 decreased the uric acid concentration (data not shown); presumably, uricase procedures with better buffering would allow the use of a higher pH, but we did not assess this.

Our experience with magnesium and inorganic phosphorus suggests that acidification would be prudent, but not as important as it is for calcium and oxalate. Reports of erroneous values for magnesium and phosphorus in alkaline infected urines (7) lead us to recommend using acidified aliquots for these analytes.

Values for creatinine tend to be lower in acidified or alkalinized samples than in untreated urines. This influence of pH has been predicted from studies of aqueous solutions (19, 20), but to our knowledge it has not been reported for urine samples.

On the basis of our preliminary data and those of Hodgkinson (7), acidification of urine to pH 1.5 will provide accurate values for oxalate but pH 2.5 to 3 will not.

In summary, erroneous values may be obtained for calcium, oxalate, and uric acid in urine specimens collected without added acid or base. Separate urine collections in acid or alkali can provide for accurate determinations, but will necessitate multiple collections of urine. Instead, we recommend that a procedure of sequential acid and alkali treatment with heat be used for urine specimens collected without preservatives. We recommend assay of calcium, oxalate, magnesium, and phosphorus in the acidified sample, uric acid in the alkalinized sample, and creatinine in an untreated aliquot. This laboratory handling of urine sample should lead to more nearly accurate values, for both clinical and research purposes, at far less inconvenience and cost to the patient.

References
3. Coe FL. Treated and untreated recurrent calcium nephrolithia-


Liquid-Chromatographic Determination of Eight Tri- and Tetracyclic Antidepressants and Their Major Active Metabolites

Tamara A. Sutfin, Robin D'Ambrosio, and William J. Jusko

A "high-performance" liquid-chromatographic method is presented for monitoring the therapeutic concentrations, in plasma, of eight tri- and tetracyclic antidepressants (amitryptiline, nortriptyline, imipramine, desipramine, doxepin, clomipramine, maprotiline, and protriptyline) and their major active metabolites. One of two internal standards is added to 0.5 mL of drug-containing plasma, the pH is adjusted to about 9.5 with borate buffer, and the sample is extracted with isooamy alcohol in hexane. The extracts are chromatographed on a column of silica and absorbance of the effluent is measured at either 214 or 254 nm. Chromatographic response is linearly related to concentration for all components over a 5-500 ng range. Analytical recovery of the drugs and metabolites from plasma is approximately 75 to 85% at low and high concentrations. Within-run and day-to-day precision (CV) is <5% for both high and low concentrations of most of these drugs and metabolites. Parallel analysis of clinical samples by gas chromatography indicates that results by the two techniques are comparable. We report some results of therapeutic monitoring of clinical samples.

**Additional Keyphrases:** monitoring therapy · drug assay · gas chromatography

The increased interest in monitoring the concentrations of tri- and tetracyclic antidepressants in plasma, and in the potential clinical significance of active drug metabolites, indicates the need for sensitive and specific assays capable of measuring, concurrently, several antidepressants and their major active metabolites. Scoggins et al. (1) reviewed 59 gas-chromatographic, "high-performance" liquid-chromatographic, and radioimmunoassay methods of analysis for tricyclic antidepressants. Only eight of the procedures can measure more than one antidepressant, and three-fourths of the 48 chromatographic methods require 2 to 5 mL of plasma. The well-known potential problem of cross reactivity between parent drug and metabolites in RIA can be particularly significant when the metabolite exhibits a different pharmacological activity and (or) potency relative to the parent drug. More recently, at least two methods, one liquid-chromatographic and one gas-chromatographic, have been reported for simultaneous analysis for several antidepressants and metabolites (2, 3). The gas-chromatographic method requires a nitrogen-sensitive detector and derivatization; the liquid-chromatographic method involves reversed-phase and requires that column temperature be kept high. Neither method includes the separation and identification of the active hydroxy metabolites of both amitryptiline and imipramine.

We previously reported a liquid-chromatographic method for assay of imipramine, desipramine, and their 2-hydroxy metabolites (4). This method has now been modified and extended to include the analysis of eight additional drugs and metabolites. It is applicable for therapeutic drug monitoring and for screening for toxicity in overdose cases.

**Materials and Methods**

**Apparatus**

The chromatographic system consists of a Model 6000A solvent delivery system, a Model U6K injector, a Model 441...