Measurement of Urinary Oxalate and Citrate by Capillary Electrophoresis and Indirect Ultraviolet Absorbance

Ross P. Holmes

We describe a method for measuring urinary oxalate and citrate with capillary electrophoresis (CE) and indirect ultraviolet absorbance detection. Sample preparation is minimal, requiring an acidification, brief centrifugation, and dilution. The method is rapid, with oxalate and citrate having mean migration times of 4.02 and 4.50 min, respectively. The minimal detectable concentration (signal-to-noise ratio of 7) of both oxalate and citrate in urine was 7 mg/L. Total imprecisions (CV) were 1.2–5.8% for three urine samples with oxalate and citrate concentrations of 8–60 mg/L and 80–860 mg/L, respectively. The recovery of added oxalate ranged from 94% to 101%. Results of CE analyses agreed well with enzymatic determinations of oxalate and citrate. Rapid analysis time, accuracy, and reproducibility make this procedure well suited for routine urinary oxalate and citrate determinations.

Indexing Terms: urine/enzymatic methods/methods comparison/chromate/chromophoric electrolyte

Analyses of urinary oxalate and citrate are important clinically in the evaluation and treatment of patients with calcium oxalate nephrolithiasis (1, 2). The measurement of oxalate in urine has proven difficult because of the much higher concentrations of other anions that interfere with direct analytical methods, and because of the presence of inhibitors in urine that interfere with enzymatic methods. A variety of procedures have been developed for the measurement of urinary oxalate, including gas chromatography, reversed-phase HPLC, ion-pairing HPLC, ion chromatography (IC), enzymatic methods, and colorimetric procedures. These have recently been reviewed by Sharma et al. (3). Citrate is routinely measured by enzymatic procedures (4) or by IC (5).

Capillary electrophoresis (CE) is an attractive method for the measurement of urinary anions (6). In this technique, anions injected as a small plug into thin capillary glass tubing move rapidly towards an anode generated by a negative power supply. This movement is enhanced by the use of a modifier of electroosmotic flow, which results from electrolyte interactions with the charge that builds up on the capillary wall. In comparison with IC, another technique commonly used for anion measurements, CE equipment is less costly and simpler to use, and running costs are much lower; assays are also generally more rapid. Because of the need for rapid acquisition of data, a microcomputer with appropriate software is required for CE analyses; however, a lower-cost integrator may suffice for IC measurements. As an analytical technique CE is sensitive, reproducible, rapid, and automated and, because of the large number of theoretical plates obtained, is able to detect major peaks with a minimum of peak tailing and peak distortion. This is especially advantageous for the analysis of oxalate in urine, where other anions such as chloride, sulfate, and phosphate are present in much higher concentrations.

Wildman et al. (6) have previously shown that CE is capable of separating urinary anions by using indirect ultraviolet absorption as a detection mode and chromate as the electrolyte. In indirect absorbance detection a chromophoric electrolyte is used and its displacement by analytes in the detection window produces a negative peak. Peak symmetry and sensitivity are optimal when the chromophoric electrolyte has a mobility similar to the ions of interest (7). Chromate is ideally suited for detecting rapidly migrating anions (8), and pyromellitic acid, a polycarboxylic acid, has also been successfully used (9). The advantage of using indirect absorbance detection is that it acts as a universal detection mode, permitting the identification of ions with a low absorptivity. A disadvantage is that the mobility of an ion will affect its peak area (10, 11), requiring that stability of migration times and variability in conductivity of sample matrices be carefully considered in developing analytical procedures. Anions resolved in urine by Wildman et al. (6) include chloride, sulfate, citrate, phosphate, and carbonate. Nitrate and oxalate, however, were not well separated. In this report, sample preparations and electrophoretic conditions required for an improved resolution of nitrate and oxalate and for the simultaneous analysis of oxalate and citrate in urine by CE are defined and the performance of the assay is evaluated.

Materials and Methods

Reagents. Analytical reagent-grade chemicals were used throughout these experiments. Sodium chromate was obtained from Aldrich (Milwaukee, WI) and tetradecylammonium bromide (TTAB) from Sigma (St. Louis, MO).

Samples. Twenty-four-hour urine collections were obtained from normal individuals and from those who had formed kidney stones, with 10 g of boric acid added to the containers as a preservative. All studies with human subjects were approved by the Institutional

Department of Urology, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC 27117. Fax 910-716-5711; E-mail rholmes@ianet.is.wfu.edu.

1 Nonstandard abbreviations: CE, capillary electrophoresis; IC, ion chromatography; and TTAB, tetradecylammonium bromide.

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Review Board. Aliquots of these collections were acidified by adding concentrated H₃PO₄ (10 mL/L urine), which reduced the pH to 2–2.5. If samples were not assayed immediately, they were stored at −20 °C. Such samples were heated at 55 °C for 30 min before analysis to dissolve calcium oxalate crystals, which were found to form under these storage conditions in some samples. Alternatively, sample dilution before freezing obviated the need for crystal dissolution. For analysis, samples were diluted 100-fold with deionized water (>18 MΩ). This dilution factor can be altered if an unsatisfactory electropherogram is obtained because the sample is overly dilute or concentrated.

**Equipment.** A Waters (Milford, MA) Quanta 4000 CE system was used with a negative power supply, a Hg lamp, and a filter kit for detection at 254 nm. Separations were obtained on a polyimide-coated fused-silica capillary (Polymer Laboratories, Phoenix, AZ) 60 cm long × 75 μm (i.d.), at a constant current of 25 μA. Data was acquired on a microcomputer with Millenium® software from Waters.

**Electrophoresis.** The electrolyte solution contained 10 mmol/L sodium chromate and 0.5 mmol/L TTAB; the pH (8.1) was not adjusted. It was degassed for 2 min before use by sonication under reduced pressure. Samples were loaded hydrostatically by gravity flow with a height difference of 10 cm for 100 s. Separations were conducted at a constant current of 25 μA. With this setting the voltage while samples were running was 15–18 kV. Between each sample the capillary was washed for 1 min with 0.1 mol/L KOH, 1 min with 0.1 mol/L HCl, and 2 min with electrolyte solution.

**Enzymatic oxalate and citrate determinations.** Urinary oxalate was measured enzymatically by using a modification of the Sigma kit 591 procedure, which relies on oxalate oxidase digestion of urinary oxalate and detection of the H₂O₂ produced (12, 13). In this modification the charcoal extraction is replaced with an overnight precipitation, and [¹⁴C]oxalate is included to monitor oxalate recovery. Citrate was estimated with a kit from Boehringer Mannheim (Indianapolis, IN) (14).

**Results and Discussion**

Wildman et al. (6) used an electrolyte consisting of 5 mmol/L chromate and 0.5 mmol/L electroosmotic modifier to show that CE was capable of resolving most of the major anions in urine. Oxalate and nitrate were not well resolved, as shown in the separation of a standard anion mixture (Fig. 1). This is important for the analysis of oxalate in urine, because the concentration of nitrate in normal urine is ~3 times that of oxalate (13, 14). Jones and Jandik (15) have shown that changes of selectivity in the separation of anions by CE can occur with changes in the concentration of electrolyte or electroosmotic modifier or by an alteration in electrolyte pH. When the chromate concentration was increased from 5 to 10 mmol/L, peaks were sharpened and nitrate and oxalate were well resolved (Fig. 1). An increase in the number of theoretical plates has been observed with an increase in electrolyte concentration for other ion separations by CE (16, 17). Calibration curves were constructed with known concentrations of oxalate and citrate added to a solution containing 130 mg/L NaCl, 22 mg/L Na₂SO₄, and 155 mg/L H₃PO₄. The presence of the Cl, SO₄, and PO₄ groups helped produce migration times for oxalate and citrate similar to those observed in urine. Absorbance changes were linear from 40 μg/L to 125 mg/L, and calibration curves over a normal working range are shown in Fig. 2.

In applying this technique to assays in urine, we encountered several problems, apparently related to interactions between urinary components and interactions of ions with the capillary wall. First, a dilution of urine is required for analysis, as chloride concentrations >200 mg/L distort anion peaks. With dilutions of 50-fold or less, recovery of added oxalate was incomplete in 10–20% of samples examined, particularly in 24-h urine collections with volumes <1.5 L. Also, with such dilutions in samples with high citrate concentrations, the citrate peak was not well resolved from nearby migrating peaks. A dilution of 100-fold produced a complete recovery of added oxalate and resolved citrate from other anions in all samples tested.
To maximize the oxalate peak with this dilution, we used a 100-s hydrostatic sample load. A linear response of peak area to load time was obtained with load times ranging from 10 to 100 s. In about one-third of the urine samples tested, the oxalate peak was very broad, as shown in Fig. 3. This affected the quantification of oxalate when its concentration in samples was low. The peak was considerably sharpened and quantification improved by adjusting the pH of the sample to <2.5 (Fig. 3). This was achieved by adding 10 μL of concentrated H₃PO₄ per milliliter of urine. H₃PO₄ was used rather than HCl because the additional Cl⁻ added with the HCl distorted the oxalate peak in many samples. The additional phosphate added with the H₃PO₄ did not distort the oxalate or citrate peaks, since it migrates after these anions. The only other sample preparation required was a 1-min centrifugation in a microfuge to prevent particulate matter from interfering with sample loading.

Urine as a matrix complicates CE analyses because of its variable composition. An approximate 10-fold range in ionic strength may be expected in urine samples. Because the electrophoretic mobility of ions is proportional to the conductivity of samples at a constant voltage, such differences in ionic strength will result in variable migration times. Differences in the migration time of an ion will affect its peak area, which depends on the mobility of the ion through the detection window (10, 11). The acidification of the urine helped partially in normalizing migration times, but a 0.5-min variability in the migration time of oxalate was observed (CV = 5.5%) when 20 urine samples were assayed under a constant voltage. The impact of variable migration times of anions in urine samples was assessed by varying the voltage between 10 and 20 kV. This indicated that a 1-min increase in the migration time of oxalate increased its peak area by 20%. Running samples at a constant current rather than at a constant voltage helped normalize migration times and decreased errors due to the variable composition of urine. The repetitive analysis of urine samples under these conditions indicated that increases in migration times occurred with each injection. This is most likely due to the binding of urinary components to the capillary wall and their effects on electroosmotic flow. The procedure used by Wildman et al. (6) did not include a washing of the capillary with alkali. Washing the capillary between samples with 0.1 mol/L KOH for 1 min greatly reduced any increase in migration time. Repeated injection of a urine sample under these conditions resulted in a gradual decline in the peak area of citrate, apparently due to its binding to the capillary wall. This was prevented by washing the column with 0.1 mol/L HCl for 1 min after the KOH wash. Slight increases in migration times still occurred with each urine sample injected under these conditions. A more extensive wash for 10 min with 0.5 mol/L KOH after ~ every 20 samples restored migration times. For such washing we used a vial containing water at the anode reservoir to avoid poisoning the electrolyte.

A typical urine profile obtained with this procedure is shown in Fig. 3. The identification of the oxalate and citrate peaks as well as their purity was determined by comparison of migration times with aqueous calibrators, supplementation of urine samples, and digestion of oxalate in samples with oxalate decarboxylase and citrate with citrate lyase. The precision of the assay as determined by the measurement of the intra- and interassay CVs in three urine samples with different oxalate and citrate concentrations is shown in Table 1. The mean recovery ± SD of 10 mg/L oxalate added to six urine samples was 93.6% ± 8.2%, 97.1% ± 4.7% for 20 mg/L oxalate added, and 101.0% ± 2.8% for 100 mg/L oxalate added. The mean recovery of citrate was 99.3% ± 2.6% for 100 mg/L added and 100.5% ± 2.2% for 500 mg/L added.

A comparison of the CE measurement of urinary oxalate with a modified Sigma 591 assay, which is based on oxalate oxidase (12, 13), is shown in Fig. 4.
Table 1. Precision of urinary oxalate and citrate determinations by CE.

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<tr>
<td>Mean oxalate, mg/L*</td>
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<td>Precision, %b</td>
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<td>1.6</td>
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<tr>
<td>Overall</td>
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<tr>
<td>Mean citrate, mg/L*</td>
<td>125</td>
<td>861</td>
<td>77.2</td>
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<tr>
<td>Precision, %b</td>
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<td>0.6</td>
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<tr>
<td>Within-run</td>
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<td>Overall</td>
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* Mean of the mean value obtained on each of 5 days replicating analyses five times.

b Precision values were calculated as described by Aggarwal et al. (18).

The correlation (r) observed was 0.98, indicating a good agreement between the two methods. The measurement of citrate by CE also agreed well with its measurement by an enzymatic procedure (4), and a correlation of 0.97 was observed (Fig. 4). The detection limit for both oxalate and citrate in urine, taken as a signal-to-noise ratio of 7, was 7 mg/L. For urine samples with concentrations below this concentration, a reduced dilution could be used to bring the values within range. Several strategies have been combined in the developed procedure to attain this level of sensitivity. Compared with the procedure described by Wildman et al. (6) for urinary measurements and by Jones (8) for measurements of anion standards, the electrolyte concentration and sample loading time have been increased, the samples acidified, the voltage decreased, and the migration time increased. All of these changes favor an increase in sensitivity. Ascorbic acid, which can break down and yield oxalate at alkaline pH (19, 20), did not affect oxalate assays when added to urine samples at a concentration of 10 g/L.

An analysis of the 24-h urinary oxalate excretions in 108 stone-forming patients with this technique revealed a mean (± SD) daily excretion of 38.6 ± 20.8 mg. The range of excretions was 7.7–118 mg, with 28% of the excretions above the limit of normal (40 mg) (1). This range of values and the frequency of hyperoxaluria in a stone-forming population are similar to those reported by others (21, 22). The mean excretion of citrate by these patients determined by CE was 432 ± 226 mg, with a range of 18–1140 mg and a frequency of hypocitraturia (<292 mg) (23) of 34%. These results for citrate excretion agree with those reported by Hatch (22) and Hosking et al. (23) for stone-forming individuals. In these samples the mean migration time of oxalate was 4.02 ± 0.16 min, and of citrate, 4.45 ± 0.17 min.

The advantages of CE for urinary oxalate and citrate measurements lie with the determination of both oxalate and citrate in a single analysis, its precision, its rapidity, and the low cost of electrolyte materials and capillary columns. It is apparently free of interferences from urinary components that influence results obtained with enzymatic methods. A further advantage of CE is that it can potentially also be used to measure other important urinary anions, including chloride, sulfate, nitrate, glycolate, phosphate, and urate. The precise conditions required for the accurate and reproducible analysis of these analytes remain to be determined.

Future developments in this instrumentation may enhance prospects for the simultaneous analysis of multiple anions in urine and plasma. It is possible that the use of suppressed conductivity detection (24) or indirect fluorescence detection may increase sensitivity. An increase in sensitivity may enable the measurement of plasma oxalate by this technique.

References