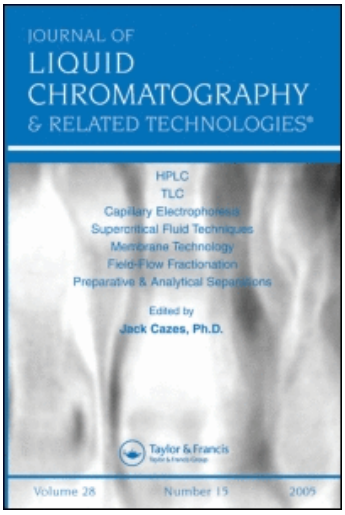


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# URINARY CITRATE ANALYSIS BY CAPILLARY ELECTROPHORESIS

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# URINARY CITRATE ANALYSIS BY CAPILLARY ELECTROPHORESIS

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## ABSTRACT

Urinary citrate is a potent inhibitor of calcium oxalate stone formation. A capillary electrophoresis method is described for the analysis of this compound. Urine was diluted in acid in the presence of an internal standard, malonic acid, and separated in a phosphate buffer 90 mmol/L, pH 6.7, for 10 min at 12.5 kV. Citrate had a migration time of about 8.5 min. The detection at 185 nm was about 7 times more sensitive than that at 214 nm. The coefficient of variance based on the internal standard was about 2 %. The method was compared to an enzymatic method and a correlation coefficient of 0.97 was observed. Both methods have some advantages as well as disadvantages.

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## INTRODUCTION

Citric acid is an important metabolite in the cell participating in the Krebs cycle. It is present in most body fluids and many types of food, such as oranges and lemons.(1) Urinary citrate is considered as a potent inhibitor of stone formation. It complexes calcium, affecting the super-saturation of urine with calcium oxalate. Patients with recurrent stones tend to have a very low concentration of citrate, both absolute and a relative (ratio) to creatinine or calcium.(2-4) Furthermore, increasing the citrate level by oral uptake in nephroliathic patients decreases the frequency of the stone formation.

Several methods are used for analysis of urinary citrate, including chromatographic and enzymatic. The most popular is an enzymatic method, which utilizes citrate lyase to cleave oxaloacetate from citric acid. Oxaloacetic acid, in turn, is coupled to NADH oxidation with the enzyme malate dehydrogenase. The amount of NADH oxidized is related to the citrate concentration(5,6). This method is available commercially as a kit. However, it is very expensive, especially when the number of samples assayed is small and occasionally gives negatives numbers.

Recently, several methods have been described for the analysis of citrate in addition to other small anions based on CE. Many of these methods utilized indirect detection.(7-9) The indirect detection in general has poor precision. Citrate has weak absorbency in the uv range but it becomes stronger as wavelengths get shorter. The aim of this work is to develop a method specific for analysis of urinary citrate based on direct detection by CE, comparing the sensitivity at the wavelength 185 nm to that at 214 nm, and also comparing the CE to the enzymatic method.

## EXPERIMENTAL

### Capillary Electrophoresis

The separation was performed in a Quanta CE instrument (Waters) at 12.5 kV with a capillary 50 cm X 75 μm. The wavelength was set at 214 nm or 185 as specified. The run time was 10 min. The electrophoresis buffer was made of 0.7 g K2HPO4, 0.7 g, KH2PO4, and 200 mg Accupure Z1 methyl reagent (Waters, Milford, MA, USA) dissolved in 100 mL water, pH 6.7. Citric acid, 1000 mg/L was used as a standard. Malonic acid, 50 mg was dissolved in 100 mL of 0.12 mol/L HC and used as an internal standard.

Equal volumes (100 μL) of urine and the internal standard were mixed and injected for 30 s (2% of the capillary volume).

### Enzymatic Method

For comparison, the commercial kit Cat 139076 (Boehringer Mannheim, Germany) was reconstituted according the manufacturer instructions and used on an automated instrument, Spectrum (Abbott Diagnostics, Abbott Park, IL). In brief, 10 μL sample was added to 250 μL of the glycylglycine buffer and 250 μL of water. The absorbency was read. After 5 minutes, 10 μL of the citrate lyase reagent was added, followed by 5 min of incubation and a second reading was obtained. The difference between the two readings was used for calculating the concentration of the citrate.

## RESULTS AND DISCUSSION

Figure 1 illustrates an electropherogram of a standard and patient analyzed at 214 nm and at 185 nm. The sensitivity at 185 nm is about 7 times higher than that at 214 nm. At both wavelengths, the assay has adequate sensitivity for clinical work and without interference from other common acids present in the urine. The internal standard, malonic acid, has a slightly different peak shape, which is helpful for identifying. The migration time for the internal standard and the citrate is about 8.5 and 9.5 min, respectively. Succinic acid migrates shortly after the citrate, with a migration time of about 10 min, Fig 1. Since we performed several other tests at 214 nm, we elected to use this wavelength for routine work in order to avoid frequent lamp and filter changes.

The peak shape and the reproducibility in CE are quite dependent on the sample matrix, among other factors. Urine samples, unfortunately, vary greatly in their pH and salt content. In order to avoid this source of imprecision, samples were diluted with HCl to avoid the differences in pH. This low pH was found, also, to induce sample stacking, especially when the sample volume is relatively large (> 20s), similar to that of field amplified stacking, Fig 2. In fact, most of the peaks in the urine, including citrate and succinate, increased in height with sample acidification. Fig 3 shows a good relationship between the sample size and peak height (up to 50 s). The ratio of citrate to the internal standard remained almost constant. This indicates, again, that some degree of stacking takes place, based on the pH difference between the sample and the electrophoresis buffer. Increasing the sample size in urine over 30 s increased the peak height, but also decreased, to some extent, the resolution. Thus, a 30 s injection was used for routine analysis.

The coefficient of variance of the analysis is dependent on the inclusion of the internal standard in the calculation and on the sample size. The CV improved with increasing the sample size up to 50 s injection. At 30 s injec-

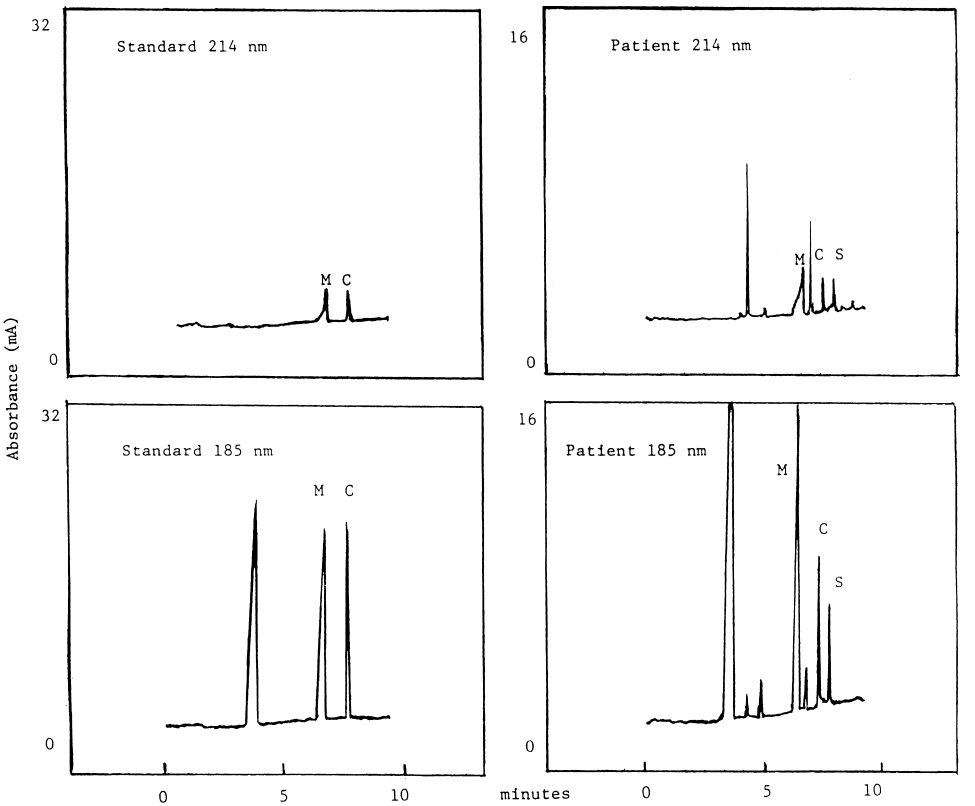


***Figure 1***. A) Standard of malonic acid, M (500 mg/L) and citric acid (1000 mg/L), C = standard with detection at 214 nm; B) Patient at 214 nm; C) same as A but detection at 185nm; and D) same as B but with detection at 185 nm (C= citric acid, S= Succinic acid, M= malonic acid).

tion, the CV based on the citrate peak alone was 10.1%; however, when the ratio of the citrate peak to the internal standard is used the CV drops to 2% (n=10).

The analysis was linear, between 50-1000 mg/L with r = 0.99, Fig 4. Succinic acid was also linear in the range of 50-1000 mg/L. The average recovery of a 500 mg/L standard added to pooled urine was 103 %(n=3). The reference interval for citrate in the urine, based on mean and 2 standard deviations for 30 normal individuals is 200-700 mg/g creatinine, which is very close to the reported range by other workers.(4,10)

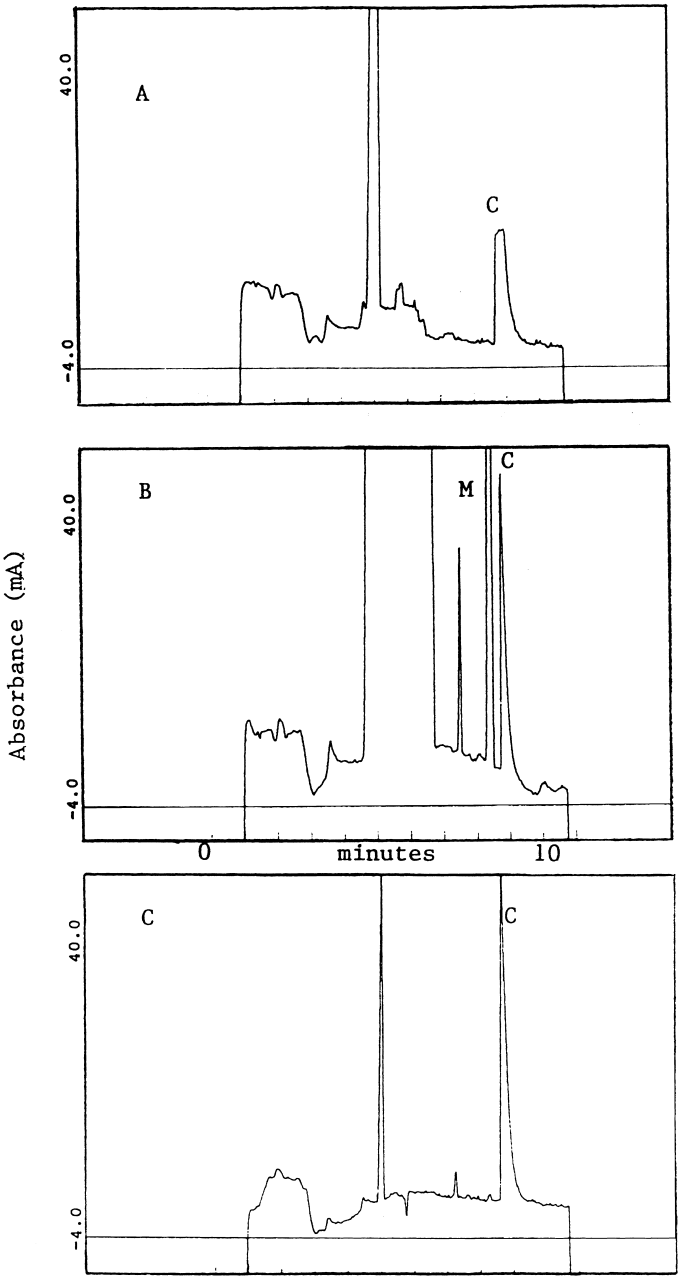


***Figure 2***. Effect of the dilution on the peak height. Sample diluted with: A- Same electrophoresis buffer, B- Hydrochloric acid, 0.12 mol/L containing the internal standard malonic Acid, m, and C- Electrophoresis buffer after being 10 times diluted (Note in A and C no internal standard is added).

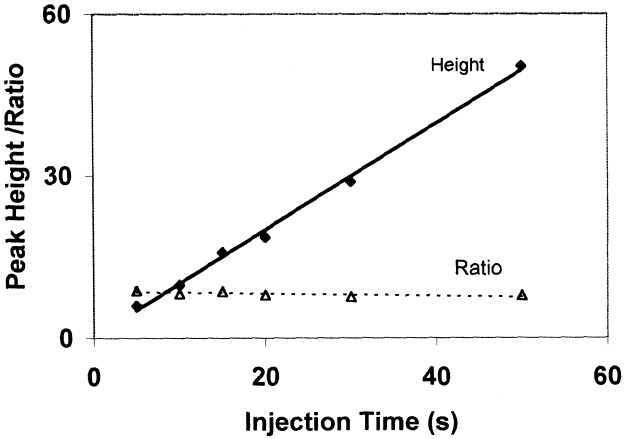


*Figure 3*. The relationship between the sample size and peak height for citrate, and as a ratio to the internal standard.

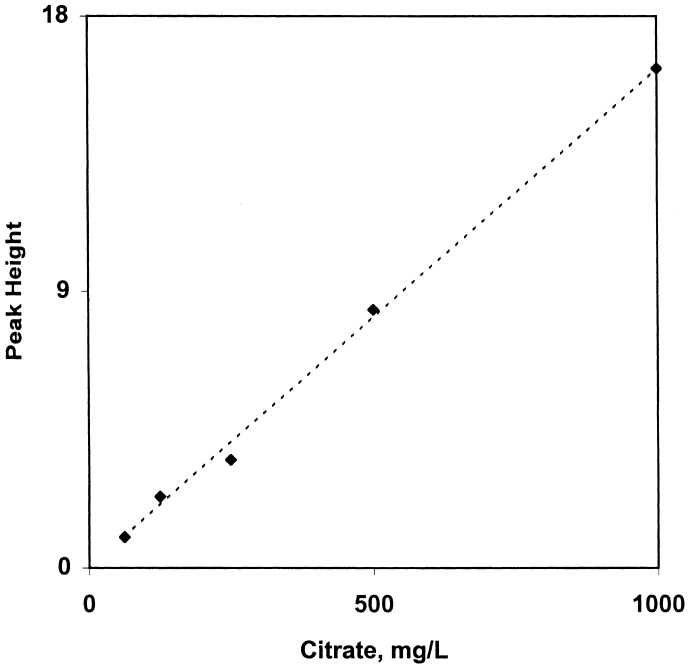


*Figure 4*. Linearity of citrate analysis by CE.

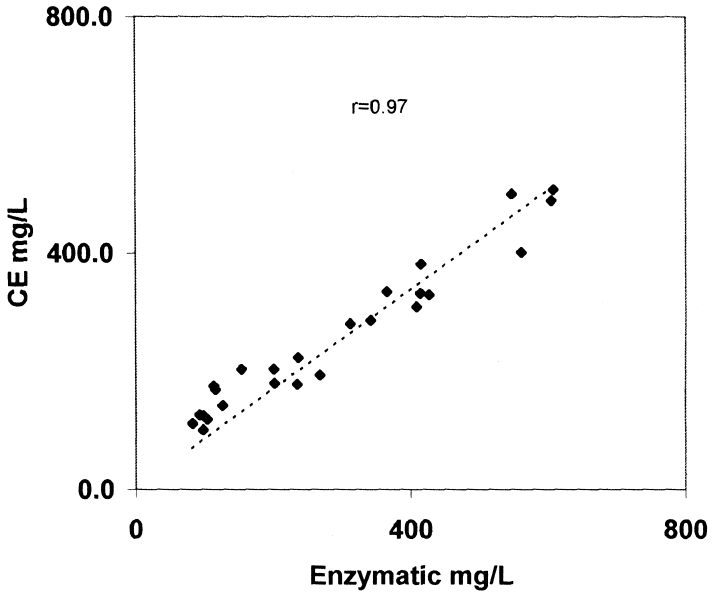


***Figure 5***. Correlation between the enzymatic method and the CE method (r=0.97).

Figure 5 shows a good correlation between the enzymatic method and the CE method with a correlation coefficient of 0.97. Both methods have certain advantages, as well as, disadvantages. Both methods require 10 min for analysis. However, the enzymatic method offers fast throughput at reasonable cost/test for a large number of samples, especially when performed on the fully automated instruments. In the enzymatic method, some samples produce negative values, especially for those with low citrate levels, probably due to interferences with the spectra of the NADH. The CE method is slower, but it does not suffer from the problem of negative numbers, and it is much less expensive compared to the enzymatic method, especially when the number of assayed samples are small. Furthermore, other acids, such as succinic can be measured in the same run by the CE method.

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