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A Simple Modified Method for Urine Citrate Determination

[İdrar Sitrat Ölçümü İçin Kolay, Modifiye Bir Yöntem]

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ABSTRACT

Objective: Measurement of urine citrate is used to assess the risk of urinary stone formation. We attempted to perform a modified, cheap and reliable colorimetric method for the analysis of urinary citrate and to compare it with an enzymatic method.
Methods: Urine citrate levels were measured with a colorimetric method and a commercially available enzymatic method in patients with urolithiasis (n=50) and in healthy controls (n=44). We modified the colorimetric method which was first developed by Millan with a subsequent modification of Lewis. Performance characteristics of the methods were compared.
Results: Urine citrate levels of patients were lower as compared to controls by both of the methods. However, the difference between patients and controls was insignificant by enzymatic method, whereas significant by in-house method ($P \leq 0.05$). Within-run imprecisions for colorimetric method were 2.1%, 3.06% and 0.52% and 3.19%, 0.91% and 2.99% for enzymatic method in low, intermediate and high citrate containing urinary pools; between-day imprecisions of the methods were 11.16%, 14.74%, 9.36%, and 17.45%, 19.94%, 23.93% in the same pools, respectively. Both of the methods were linear up to 5 mmol/L. The detection limits of colorimetric and enzymatic methods were 0.19 mmol/L and 0.357 mmol/L; mean recoveries were 88.7% and 89.46%, respectively. The coefficient of correlation between the methods was r=0.922.
Conclusion: Colorimetric method is superior to the enzymatic method. Colorimetric method more efficiently detects lower urine citrate levels in urolithiasis patients and discriminates patients from controls.
Key Words: Citrate, urolithiasis, urine stone disease, method comparison

ÖZET

Amaç: İdrar sitrat ölçümü, üriner taş oluşumu riskini değerlendirmek için kullanılır. Çalışmamızda üriner sitrat analizi için; yeni, ucuz ve güvenilir kolorimetrik yöntem geliştirmeyi ve bunu enzimatik yöntemle karşılaştırmayı amaçladık.
Yöntem: İdrar sitrat düzeyleri, üriner sistem taş hastaları (n=50) ve sağlıklı kontrollerde (n=44) kolorimetrik yöntem ve ticari olarak kullanılan enzimatik yöntemle ölçüldü. Millan tarafından geliştirilip Lewis tarafından modifiye edilen kolorimetrik yöntemi modifiye ettik. Yöntemlerin performans özellikleri karşılaştırıldı.
Bulgular: Hastaların, idrar sitrat düzeyleri kontrol grubuyla karşılaştırıldığında her iki yöntem ile de düşük bulundu. Bununla birlikte, hasta ve kontrol grupları arasındaki fark enzimatik yöntemde anlamsız iken geliştirdiğimiz yöntemde anlamlıydı ($P \leq 0.05$). Çalışma içi tekrarlanabilirlik, düşük, orta ve yüksek sitrat içeren idrar havuzlarında kolorimetrik yöntem için sırasıyla %2.1, %3.06, %0.52; enzimatik yöntem için %3.19, %0.91 ve %2.99; yöntemlerin günler arası tekrarlanabilirliği ise aynı havuzda, sırasıyla; %11.16, %14.74, %9.36 ve %17.45, %19.94, %23.93 idi. Her iki yöntem de 5 mmol/L' ye kadar lineer idi. Saptama sınırı, kolorimetrik ve enzimatik yöntem için, sırasıyla 0.19 mmol/L ve 0.357 mmol/L; ortalama geri kazanım %88.7 ve %89.46 olarak bulundu. Yöntemler arasındaki korelasyon katsayısı r=0.922 idi.
Sonuç: Kolorimetrik yöntem enzimatik yönteme göre daha üstündür. Kolorimetrik yöntem, üriner sistem taş hastalarında daha düşük idrar sitrat düzeylerini tesbit etmede ve kontrollerden hastaları ayırt etmede çok daha yararlıdır.
Anahtar sözcükler: Sitrat, üriner sistem taş hastalığı, idrar taş hastalığı, yöntem karşılaştırma

Introduction

Urinary system stones form in renal pelvis, urether or urinary bladder. Calcification may also form in renal parenchyma which is called nefrocalcinosis. Presence of stones results in tissue loss, lack of function, hematuria, infection and obstruction. Renal colic is one of the main conditions for admitting emergency services [1].

Many reasons that have effects on the balance of inhibiting and stimulating factors contribute to formation of urinary stones [2]. Urinary citrate concentration plays an important role in formation of calcium oxalate and calcium phosphate stones in urinary tract. Citrate present in urine forms soluble complexes with calcium leading in decrease of supersaturation of calcium oxalate and calcium phosphate. It is thought that inhibitory effect of citrate has a role in prevention of formation of urinary stones. Hence, serum and urine citrate levels draw interest in urinary tract stone disease. In many studies involving patients with urinary tract stone disease the excretion of citrate is significantly low [3].

Recently, citrate determination in urine is mainly carried out by enzymatic methods. In this study it is aimed to develop a new, cheap and reliable colorimetric method and to assess its performance characteristics while comparing it with the enzymatic method.

Materials and Methods

Patient and Control Groups

Patient group consists of 28 male (mean age 33.7±13.2 years) and 22 women (mean age 34.9±14.3 years) in a total of 50 patients with urolithiasis. Control group consists of 23 male (mean age 40.6±13.1 years) and 21 women (mean age 43.5±16.5 years) in a total of 44 healthy volunteers. From both patient and control groups, 24 h urine samples were aliquoted after collection and stored at -84 ^{o}C until analysis. The study was approved by Local Ethics Commitee of our hospital.

Enzymatic Measurement of Citrate

Enzymatic citrate levels were determinated by using commercially available reagents (FAR srl, Verona, Italy). Citrate converted to oxolacetate and acetate by catalytic activity of citrate lyase. In the presence of L-malate dehydrogenase and L-lactate dehydrogenase, oxaloacetate and its decarboxylation product pyruvate are reduced into L-malate and L-lactate by NADH. The amount of oxidized NADH level is correlated with the level of citrate in the sample. Citrate concentration is calculated by the decrease of absorbance at 340 nm. The method was adapted to Olympus AU 400 analyzer.

Colorimetric Measurement of Citrate

We modified the method which was first developed by Millan [4] with a subsequent modification of Levis [5]. In alkaline pH, phosphates in the urine were precipitated by MgCl (E. Merck, Darmstadt, Germany) and citrate forms a yellow colored complex which can be monitored by spectrophotometrically at 390 nm. Citric acid trisodium salt (Sigma Chemical Co., USA) was used as standard in the study.

Equipment

Citrate measurements were performed on a Shimadzu CL-770 spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

Reagents

Instead of 30% NH_4OH solution described by Millan et. al. [4], 25% NH_4OH solution was used.

Concentration of Magnesium chloride solution was 0.2 mol/L and HCl solution was 10 mol/L (pH=2) .

$FeCl_3$ solution, 18 mmol/L, was prepared freshly, by deionized water instead of HCl solution.

Procedure

Urine specimens were kept frozen (- 84 °C). Before analysis, the samples were brought to room temperature, then mixed. 0.1 mL NH_4OH (%25) was added to 4 mL of sample and was mixed well by vortex. 0.9 mL $MgCl_2$ solution was added and mixed on a vortex mixer and the mixture was centrifuged at 4000x g for 10 min to obtain phosphate-free urine; then, supernatant was transferred to clear tubes. After adjusting pH of supernatant to 2 with 0.1 mL 10 mol/L HCl, supernatant was again mixed by vortex. 0.25 mL of $FeCl_3$,18 mmol/L, was added to the mixture and mixed on a vortex mixer, absorbances were immediately read against the deionized water at 390 nm with a spectrophotometer.

Reagent blank was prepared with the addition of 0.25 mL of $FeCl_3$ to 4.75 mL pH 2 HCl solution and read against the deionized water.

Urine blank was prepared with the addition of 0.75 mL of urine sample to 4.25 mL pH 2 HCl solution and read against the deionized water.

Five solutions of citrate (0.312, 0.625, 1.25, 2.5 and 5.0 mmol/L) were prepared in deionized water for every study and used as standarts.

Performance Characteristics of Colorimetric and Enzymatic Measurements of Citrate

a. Imprecision studies

For estimating within-run and between-day precision, 3 different sample pools were generated with low, medium and high levels of citrate. For within run precision, all of the pools were assayed 21 times at the same serie. For the day to day precision samples from all different pools were aliquoted and stored at -84 °C. In each day lasting for 21 days, one sample representing each pool were thawed and assayed. Mean (X), standard deviation (s) and % coefficient of variation (% CV) were calculated.

b. Linearity studies

A solution of citrate, 10 mmol/L, was prepared. With serial dilutions samples with 10, 7.5, 5, 3.75, 2.5, 1.25, 0.625 and 0.312 mmol/L of citrate were prepared and assayed in duplicate by two methods, simultaneously.

c. Recovery studies

For recovery studies, known amount of citrate were added into the known level of citrate in the sample pool. All the samples were assayed in duplicate and percent recovery was calculated at five different levels.

d. Detection limit studies

To determine the detection limit of the methods, blank readings were carried out 21 times without adding the sample. Avarage and standart deviations of absorbance values were calculated. Border of detection was determined by adding standart deviation x 2 to the average blank absorbance.

e. Color stability

We checked the stability of reaction product by measuring absorbance changes with 10 minute intervals. The reaction absorbance remained the same up to 100 min. Additionally, absorbance values did not change also neither increasing nor decreasing way by incubation at 37 ^{o}C for 100 min.

Statistics

All statistical analyses except Deming regression analysis were performed by SPSS® for Windows 15.0 (SPSS Inc. Headquarters, Chicago, lll., USA) software program. Deming regression analysis was performed by Analyse- it statistical program.

Results

Urine citrate values in patient and control groups are shown in Table l.

Colorimetric citrate ($Citrate_{COL}$) and enzymatic citrate ($Citrate_{ENZ}$) methods were compared according to their performance characteristics such as the imprecision, linearity, detection limits and recovery**.** The lineer regression analysis of two methods was also compared to each other.

Imprecision study was performed at three different citrate levels; within-run imprecisions were 2.1%, 3.06% and 0.52% for colorimetric method and 3.19%, 0.91% and 2.99% for enzymatic method; between-day imprecisions of the methods were 11.16%, 14.74%, 9.36%, and 17.45%, 19.94%, 23.93%, respectively. According to these results, colorimetric method gave much better results in both within-run and betwen-run imprecision studies.

Linearity study showed that linearity was similar in both methods and they were linear up to 5.0 mmol/L of urinary citrate concentration.

A strong correlation was obtained between colorimetric and enzymatic citrate methods (r=0.922) with regression analysis (Figure 1). Regression equation was:

y (colorimetric) = 0.741(CI:0.537-0.945) + 0.851(CI:0.777-0.925) enzymatic

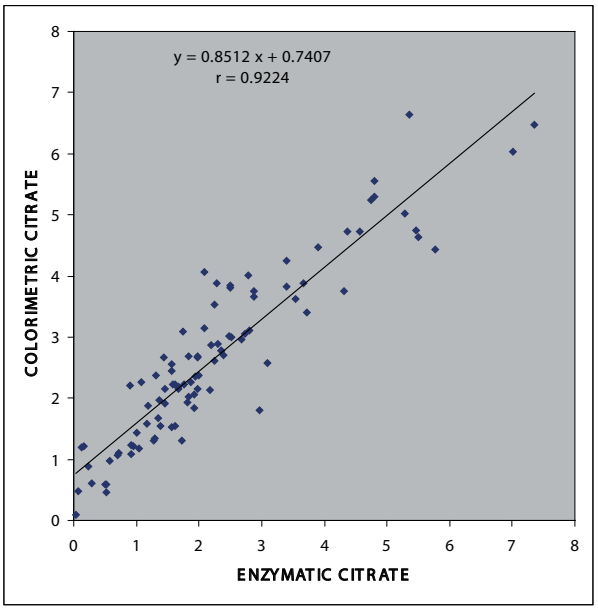

Figure 1. Deming's lineer regression analysis of $Citrate_{ENZ}$ and $Citrate_{COL}$ methods.

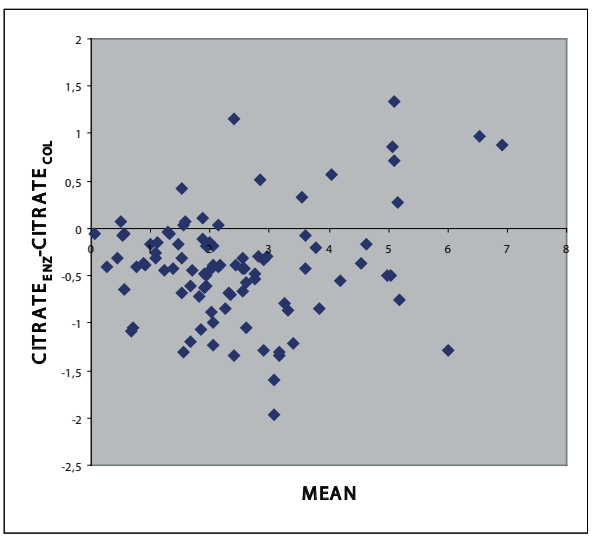

Figure 2. Bland - Altman graph of $Citrate_{ENZ}$ and $Citrate_{COL}$ methods. $Citrate_{ENZ}$ gave slightly lover citrate concentrations than $citrate_{COL}$.

Table 1. Urine citrate values in patient and control group

Analyte	Patient (x ± SD)	Control (x ± SD)
Urine $citrate_{ENZ}$ (mmol/day)	2.0 ± 1.6	2.56 ± 1.33
Urine $citrate_{COL}$ (mmol/day)	2.30 ± 1.4 *	3.10 ± 1.2 *

Statistically significant at *P<0.05 level.

The detection limit of colorimetric method was found to be 0.19 mmol/L, whereas detection limit of enzymatic method was 0.356 mmol/L. Detection of lower levels of citrate is important in clinical practice. Thus, colorimetric method is better in the detection of lower levels of citrate for clinical purposes (Figure 2).

Recovery study showed that R% values obtained in both methods were quite similar. It was observed that mean R% for colorimetric and enzymatic methods were 88.7% and 89.46%, respectively.

Discussion

By this procedure following methodological developments were reached:

The preparation of sample blank was corrected;

Because of the large pH variations after the addition of $FeCl_3$ reagent (18 mmol/L $FeCl_3$ in 1 mol/L HCl), we modified preparation of $FeCl_3$ reagent by dissolving the $FeCl_3$ in deionized water instead of HCl.

pH of the reaction mixture is very important for the color intensity of citrate- Fe^{3+} complex. Therefore, we adjusted the pH of reaction mixture to 2.0 for every sample.

Sample to reagent ratio was optimized. We used 750 µL dephosphatized sample instead of 4.75 mL.

Method performance studies showed that colorimetric method was superior to enzymatic assay, in general. Especially, lower detection limit and CV % at low citrate concentrations are advantages of colorimetric assay, as lower citrate concentrations are clinically more important.

The original method of Millan et al [4] had been severely criticized by Top and Yucel [6] because of erroneously prepared sample blank, detection limit and linearity of the method. Lewis [5] improved the method by use of 250 uL dephosphatized urine instead of 4.75 mL. However, Lewis reported that the method cannot be used at concentrations of citrate < 2.5 mmol/L. In the preliminary studies of our investigation, we used 0.25, 0.5, 0.75 and 1.0 mL of dephosphatized sample and obtained the optimal absorbance values (analytical sensitivity) with 0.75 mL of it.

One of the main contributing factors on urinary system stone formation is urine citrate level. Because of the wide and different range of citrate excretion in different studies results of the present study are reasonable. The values reported for healthy subjects are 174.7 mg/day (range 73.8 to 378.4 mg/d); 4.05 ± 1.22 (range 2.12 – 6.26) mg/d; 643 mg/d; 2.2 – 4.4 nmol/d; 1.6 – 4.5 mmol/L; 76 – 792 mg/d; and 2.29 mmol/d (range 0.91 – 3.81) [7-12].

Many articles have reported that urinary citrate excretion rate in patients with urinary tract stone disease are significantly lower than that in control groups [13-17]. In our study, we found that urine citrate levels of patients were lower as compared to controls by both of the methods. However, the difference between patients and controls was insignificant by enzymatic method, whereas significant by in-house method ($P<0.05$).

Hypocitraturia incidence in stone formers was reported as 34% in a study [18]. In the present study, we found hypocitraturia incidence as 26% and 22% by enzymatic and colorimetric methods, respectively. Hypocitraturia rates in controls were 4% and 2%.

In conclusion, colorimetric citrate method was found to be better than enzymatic method according to analytical and clinical performances. Colorimetric method more efficiently detects lower urine citrate levels in urolithiasis patients and discriminates patients from controls. Both methods can be applied to routine clinical laboratory practice without the presence of more complex equipments. Enzymatic method can be fully automatized. But, colorimetric method can only be automatized after dephosphatisation at complex formation stage. Citrate test is rarely studied in different laboratories. Therefore, automatization for the citrate test is not so meaningfull.

Colorimetric citrate test that we improved in our laboratory is a suitable and cheap method for routine studies as compared with enzymatic assay. The costs of colorimetric and enzymatic assays per test are 0.1 TL and 2.5 TL, respectively.

Study limitation

The pH of the reaction mixture is utmost important for the color intensity of citrate-Fe^{+3} complex. The pH can be adjusted to pH 2.0 by a buffer (e.g., glycine HCl buffer). We aim to perform this modification in the near future.

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