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A novel method for the determination of urinary citrate levels: Ion-pair and high performance liquid chromatography with liquid membrane extraction technique

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ABSTRACT

In the clinical evaluation, and treatment of the patients with urinary calcium oxalate stones, determination of urinary citric acid levels is as important as oxalic acid levels. Presence of specific matrix proteins, and other anions in urine complicates its measurement. Therefore we aimed to develop a routine high performance liquid chromatography (HPLC) method which could determine citric acid levels in urine, and other body fluids. One hundred patients referred from various clinical sections were included in the study, and their urinary citrate levels in spot or 24-hour urine samples were measured as required. Measurement of oxalic acid was performed with a liquid membrane technique using a 314 nmUV detector at a stationary phase of C18 column, under 25°C ambient temperature, and at a flow rate of 1.7 mL/min Internal standard method was used for the determination of citrate concentration. For citrate standards at two distinct concentrations (1.25 mmol/L, and 5 mmol/L) respective intraday, and interday coefficient of variation (CV) values were found to be 4.29 vs 5.37%, and 2.78 vs 3.13% with a linearity interval of 0.03-20 mmol/L. In three different concentrations mean recovery rates of 101.2, 100, and 99.7% were obtained, respectively, and functional sensitivity of the assay was determined as 0.009 mmol/L. In conclusion, thanks to this method we developed, problems encountered during analysis of biological samples with complex matrix appears to be solved at greater extent. Besides compared with other available methods, this HPLC assay yields results in a shorter time frame with higher cost-effectiveness, simplicity, reproducibility, and reliability Therefore, we think that it can be used routinely in the determination of citrate levels in urine, and other body fluids.

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1. Introduction

Citrate inhibits growth, aggregation, and nucleation of urinary crystals, and thus stone recurrence. As a strong chelating agent of calcium, citrate is one of the widely used therapeutic agent for the prevention of the formation of calcium oxalate, uric acid and cystine stones because of its capacity to alkalinize urine and decrease supersaturation of urinary calcium (Balaji and Menon, 1997; Wahl and Hess, 2000). Citrate promotes the formation of nicotinamide adenine dinucleotide phosphate (NADPH) thus contributing to the cellular antioxidant defense (Smith, 1989). In nearly 50% of the patients with calcium oxalate stones, citrate concentrations in 24-hour urine samples were detected to be lower than 2.5 mmol/L (Ansari and Gupta, 2003). Approximately 85% urinary tract stones are made of calcium, and these stones constitute the most important causes of recurrent stone disease. In the follow-up, and management of the patients with recurrent stone disease, determination of citrate excretion is very important. Indeed, urinary citrate forms soluble complexes with calcium, thus inhibiting formation of calcium salts. As a risk factor, hypocitraturia can occur because of renal tubular acidosis, chronic diarrhea, chronic diuretic use, and chronic dehidration. In these cases, the best form of therapy is administration of oral potassium citrate pills or effervescent tablets. Measurement of urinary citrate levels in order to evaluate beneficial effects of therapy, and potential risk of stone recurrence in the future guides the physicians through their clinical applications (Pearle et al., 2005)..

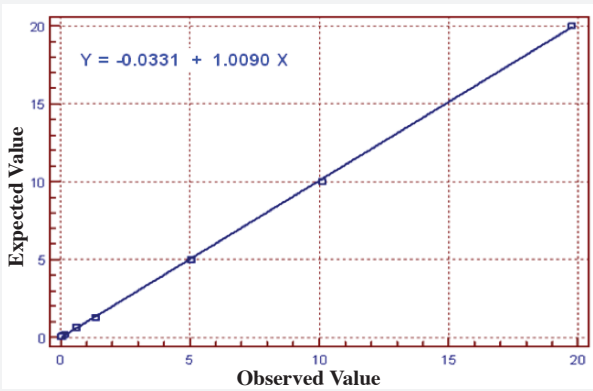

Fig. 1. Graphics demonstrating linearity of urinary citrate measurements (=0.99, p<0.001)

Nowadays, citrate assays are generally performed using time-consuming enzymatic methods requiring qualified personnel, and expensive kits. Therefore, we intended to develop an original, and user-friendly high performance liquid chromatography (HPLC) measurement method with faster turnaround times, and higher cost-effectiveness, sensitivity, and specificity.

Table 1. Intraday, and interday certainty values for the method

	Intraday		Interday	
Concentration (mmol/L)	**Mean±SD**	**CV (%)**	**Mean±SD**	**CV (%)**
1.25	1.22±0.03	4.29	1.21±0.07	2.78
5	5.03±0.01	5.37	4.9±0.18	3.13

CV: Coefficient of variation

2. Material and method

Collection of samples

One hundred patients whose urine citrate levels were monitored in clinics of Ondokuz Mayıs University Healthcare Application and Research Center were included in the study. Spot or 24-hour urine samples were collected. Twenty-four hour samples were collected in plastic urine collector bags containing 10 mL 6N HCl under a cool environment protected from light.

Analytical procedure

Five hundred microliters of urine sample was mixed with 20 μL internal standard (tartaric acid), and then 500 μL 0.01M barium hydroxide ($Ba(OH)_2$) was added on the mixture. This solution was centrifuged at 4000 xg, and the supernatant was transferred into another tube, then mixed with 500 μL tri-n-octylamin-chloroform/phosphoric acid. Two hundred microliters of 0.5 M sulfuric acid H_2SO_4 was added on the mixture, agitated for 15 minutes, and aqueous portion of the mixture was removed after centrifugation.The preparation was forced into a HPLC device using an isocratic pump at 25°C, and at a flow rate of 1.7 mL/min. A 314 nm UV detector with a column caliber of 250x4 mm containing 5 μm- particles was used in the study. Mobile phase was prepared from a mixture of 4.5 mM potassium dihydrogen phosphate (pH 2), and 2.5 mM tetrabutyl ammonium bisulfate. The results were calculated using an internal standard.

3. Results

Intraday, and interday certainty values for our citrate analysis

Table 2. Recovery rates in measurements of urinary oxalate levels

Added concentration (mmol/L)	Added concentration (mmol/L)	Measured value (Mean±SD)	Recovery rate %
2.83	10	12.99±0,19	101.2
2.83	2.5	5.33±0,01	100.0
2.83	0.65	3.44±0	99.7
Mean			100.3

are shown in Table 1. To estimate the linearity of the citrate assay, from our 20 mmol/L citrate solution, standards with various concentrations (20, 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039 mmol/L) were prepared using serial dilutions. Each standard solution were used thrice, and subjected to linear regression analyses (Fig. 1).

Besides five standard solutions with lower concentrations (0.156, 0.078, 0.039, 0.019, 0,009 mmol/L) obtained using serial dilutions were analyzed for five times, and the results were checked with non-linear regression analyses. From the above plotting, the value (0.009 mmol/L) corresponding to 20% coefficient of variation (CV) was found, and defined as cut-off value of functional sensitivity (Fig. 2).

On urine samples with known citrate concentrations, citrate solutions with three different concentrations were added, and mean recovery rate of analyses repeated three times was found to be 100.3 percent (Table 2).

On chromatograms, mean internal standard, and citrate retention times were achieved as 2 ± 0.2 min and, 3.7 ± 0.4 min, and mean total duration of analysis was 6 minutes. Analyzed calibrator chromatograms, and control chromatograms of normal, or abnormal values, and urine samples are shown in Fig. 3, 4, 5, and 6 in that order.

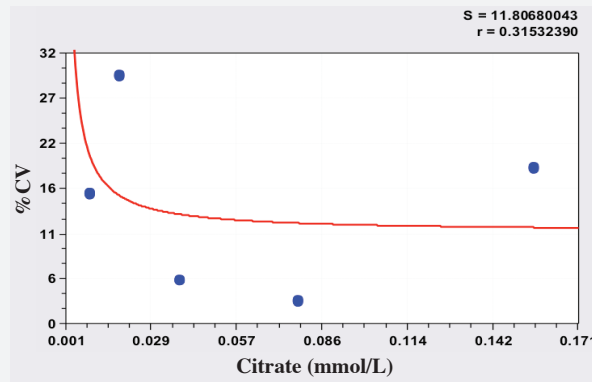

Fig. 2. Functional sensitivity chart for urinary citrate measurement method

4. Discussion

Urinary system stone disease ranks third in frequency among urinary system diseases, and its incidence in men is 2-fold higher (Amaro et al., 2005). In an investigation performed in 1989 on 1500 individuals from 14 cities of Turkey, its prevalence, and incidence were 14.8, and 2.2%, respectively with a male/female ratio of 1.5 (Akinci et al., 1991). In other countries, hypercalciuria appears to be an important risk factor, however in our country hypocitraturia is a predominant risk factor (Öner, 2009).

Nearly 80% of the stones are made of calcium oxalate, and calcium phosphate, and the rest were composed of

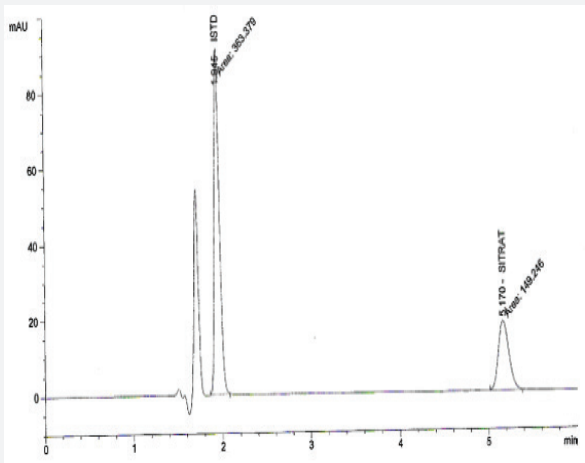

Fig. 3. Calibrator chromatogram of citrate

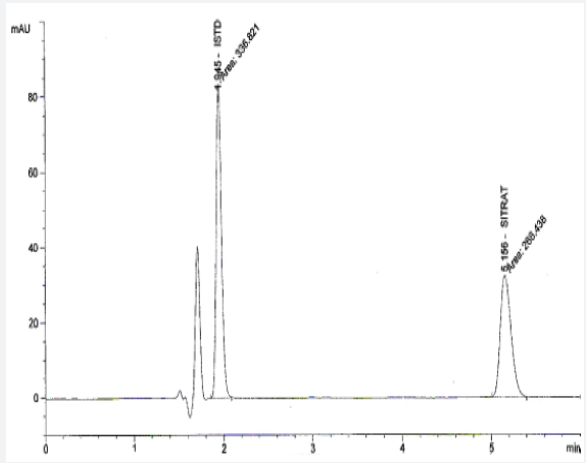

Fig. 5. Control chromatogram of abnormal citrate levels

struvite (magnesium ammonium phosphate) (10%), uric acid (%9), and cystine, ammonium acid urate, xanthine, 2,8-dihydroxyadenine, protein matrix, and drugs like indinavir and triamterene (1% all combined) (Fredric et al., 2005; Moe, 2006). Although mechanism of calcium oxalate stone formation which constitues majority of kidney stones is not fully understood, crystallization is the first step in the formation of stones. Crystallization is dependent on various factors such as urinary calcium oxalate saturation, and concentrations of substances stimulating, and inhibiting urinary crystallization. Both experimental, and clinical studies have confirmed calcium oxalate stone formation inducing effects of urinary oxalate excretion , and conversely inhibitory effects of citrate (Kok et al., 1990). Oxalate levels in urine, and urinary concentration of citrate are important risk factors for the development of stones (Holmes and Kennedy, 1999). Analysis of urinary citrate is very important for the clinical monitorization, and treatment of patients with calcium oxalate stones (Petrarulo et al., 1995).

Up to now, various methods have been used to measure urinary citrate levels including direct precipitation, colorimetric method, atomic absorption spectroscopy, enzymatic method, capillary electrophoresis, gas chromatography, HPLC, radioenzymatic isotop dilution method, and liquid chromatography tandem mass spectrometry (Beutlere and Yeh, 1959; Dempsey et al., 1960; Li and Madappally, 1989; Lewis, 1990; Holmes, 1995; Petrarulo et al., 1995; Keevil et al., 2005; Keevil and Thornton, 2006).

Nowadays, citrate assays are realized using enzymatic time-consuming methods requiring qualified personnel, and expensive commercial kits (Li and Madappally, 1989; Petrarulo et al., 1995). Enzymatic method yields lower recovery rates because of interferences by phosphate, and sulphates (Ogawa et al., 1987) At the same time, reliable but very expensive methods as capillary electrophoresis, and liquid chromatography-tandem mass spectrometry have been used (Holmes, 1995; Keevil et al., 2005, Keevil and Thornton, 2006). Rates of accuracy, and reproducibility of liquid chromatography-tandem mass spectrometry are below 3% with a mean analytical time of 4 minutes. Nowadays, in clinical laboratories, extremely sensitive, and reliable HPLC techniques have been used for the measurement, and separation of various analytes. Scarce number of literature studies have investigated citrate levels in clinical samples based on HPLC measurements. In a method suggestively developed by Khaskhali et al. (1996) urine samples were firstly deproteinized by sulfosalicylic acid, then filtrated, and citrate levels were detected in HPLC device at 210 nm within a short time of 10 minutes. On chromatograms in the relevant article, pure citrate peaks were shown, and recovery rate was found to be 102%, with intraday, and interday CV values of 1.2, and 0.2%, respectively. However, in our studies which

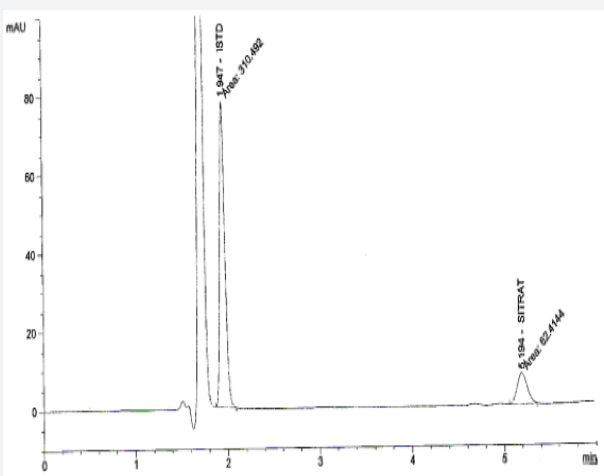

Fig. 4. Control chromatogram of normal citrate levels

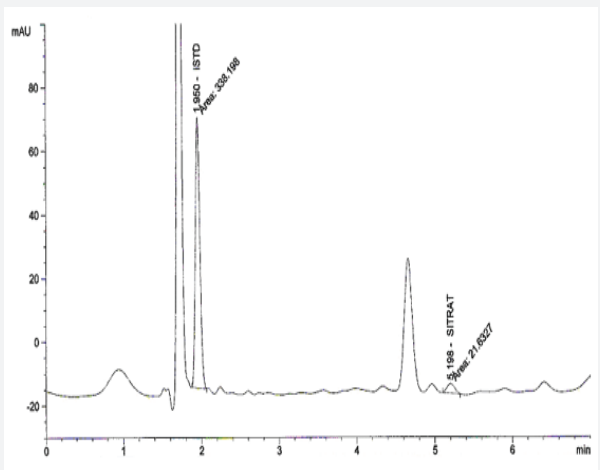

Fig. 6. Chromatogram demonstrating urinary citrate levels

referenced the abovementioned article, successful outcomes could not be obtained. In fact, citrate component could not be extracted from urine samples despite many attemps, and a pure peak could not be achieved. In spite of many trials of deproteinization using different acids, a successful outcome could not be accomplished.

Then, as a fruitful yield of our strenuous attempts, we developed an organic liquid membrane (OLM) technique. Using our OLM technique, we successfully extracted citrate from urine which contains a special matrix, and excess amounts of other anions. In our method, sulphate, and phosphate anions in urine samples were precipitated with barium hydroxide, and then 20 μL internal standard was added on the supernatant. Afterwards, this solution was incubated with OLM which composed of tri-n-octylaminechloroform/phosphoric acid mixture. After recovery of citrate components in OLM using sulfuric acid solution, HPLC analysis was performed.

As an important component in the early diagnosis, and monitorization of urinary stone diseases, citrate has been firstly measured using liquid membrane extraction technique based on HPLC analytical method. This original technique we developed is a cost-effective method with higher reproducibility, reliability, and faster turnaround times. Therefore, we think that this method is suitable for use in daily clinical practice.

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