Urol Res (1992) 20:165-168

Urological Research

A new method for determination of urinary citrate

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Accepted: August 1, 1991

Summary. We have developed a new assay technique using high-performance liquid chromatography. The assay was performed at a flow rate of 0.7 ml/min, a temperature of 60 °C and an ultraviolet absorption of 214 nm. Comparison of the results of the new assay with the results obtained for identical samples using the conventional fluorometric method demonstrated a very high correlation coefficient of 0.931.

Key words: Urinary citrate - HPLC - Urolithiasis

The chelating action of citrate acid on calcium ions results in a soluble complex salt of urinary calcium with citric acid. Citric acid is therefore considered to be a substance of importance for the prevention of calcium-containing urinary calculi [2]. The urinary citrate assay is important for clarifying the cause of urinary stone formation. Traditionally, fluorometry [4], enzymatic assay [5] and other methods [3, 6, 7] have been used for this purpose. However, none of these methods has become popular, as the assay procedures are complicated or take a long time. Until recently, we have used the fluorometric method, but we have now developed a new assay for urinary citrate, based on high-performance liquid chromatography (HPLC). This paper discusses the feasibility of the clinical application of this new method.

Materials and methods

The assay method and equipment used in the present study are illustrated in Fig. 1. A 5-ml portion from a 24-h urine sample was diluted with 15 ml 0.5% H_3PO_4. A SEP-PAC C18 filter (Waters, Milford, Mass., USA) was washed with 10 ml 0.5% H_3PO_4 before assay. The diluted urine sample was passed through the filter. A 20-µl aliquot of the filtrate was injected into an HPLC column with an autoinjector (SCL-6A, Shimazu, Osaka, Japan). The samples were warmed to 60°C with a column heater (HTR-C, Waters) and then introduced into an HPLC system pump (Model 510, Waters). Chromatograms were recorded on chart paper (0.5 cm/min) using a recorder (U-228, Nihon Denshi Kagaku, Osaka, Japan). Data were recorded on chart paper as shown in Fig. 2. The citrate peak appeared 8.5 min after injection. Chart speed was 0.5 cm/min.

The urinary citrate of 115 patients with calcium-containing calculi in the upper urinary tract (80 men aged 39.2 ± 16.5 years, 35 women aged 43.2 ± 7.8 years) and of 66 non-urolithiasis patients (38 men aged 40.8 ± 11.2 years, 28 women aged 40.6 ± 11.4 years) was determined using the HPLC method. A 24-h urine specimen from each of these patients was collected in plastic bottles without preliminary treatment, and all were kept at 4°C until the time of measurement.

The tests of significance of difference between groups were performed using Student's *t*-test.

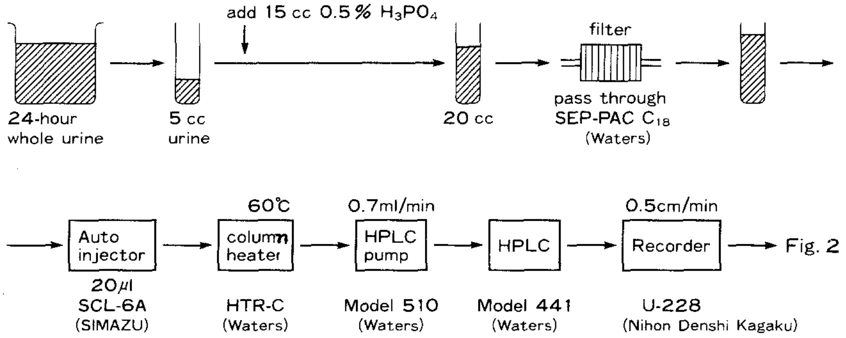

Fig. 1. Assay method and equipments

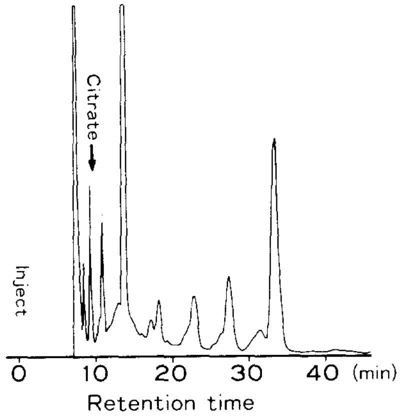

Fig. 2. Chromatogram of a urine sample

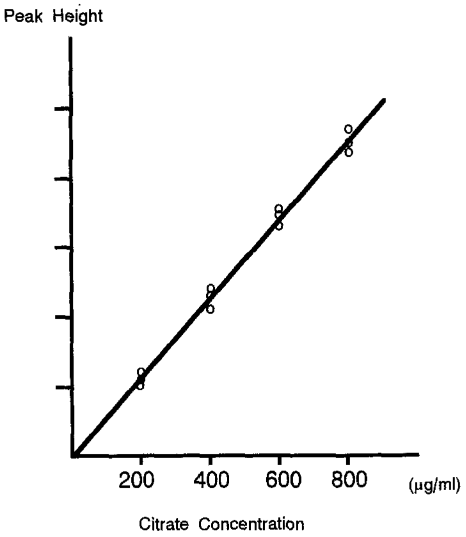

Fig. 3. Standard curve for citrate

Table 1. Reproductivity of the determination of urinary citrate. Variation coefficients in the triplicate assay were in a acceptable range of 1.7 to 4.3%

Sample No.	Citrate concentration (μg/ml)			Mean ± S.D.	C.V. (%)
	1	2	3		
1	533	544	522	533 ± 9.1	1.7
2	577	611	588	592 ± 13.8	2.3
3	777	844	788	803 ± 29.1	3.6
4	477	511	511	500 ± 15.7	3.1
5	1021	1099	1132	1084 ± 46.5	4.3
6	355	372	345	357 ± 13.7	3.8
7	644	688	650	661 ± 23.9	3.6
8	826	855	834	838 ± 15.0	1.7
9	411	465	433	436 ± 27.2	6.2
10	856	895	960	870 ± 21.5	2.5
				mean ± S.D.	3.3 ± 1.4

Table 2. Recovery rate of citrate added in urine. Recovery rates after addition of citrate at 200 μg and 400 μg to urine were 101.5 ± 6.3% and 96.0 ± 2.6%, respectively

	Sample No.	Citrate Concentration (μg/ml)			Recovery Rate (%)
		Original Urine	Added Urine	Added-Original	
200 μg added	1	428	647	219	109.5
	2	539	736	197	98.5
	3	517	723	206	103.0
	4	433	615	182	91.0
	5	556	767	211	105.5
	6	644	850	206	103.0
	7	826	1034	208	104.0
				mean ± S.D.	102.1 ± 5.9
400 μg added	1	428	832	404	101.3
	2	539	916	377	94.3
	3	517	893	376	94.0
	4	433	817	384	96.0
	5	556	934	378	94.5
	6	644	1017	373	93.3
	7	826	1197	371	92.8
				mean ± S.D.	95.2 ± 2.9

Table 3. Dilution recovery rate of urine. Recovery rates at twofold and threefold urine were 96.8 ± 101.2% and 94.6 ± 101.9%, respectively

Sample No.	Citrate Concentration (μg/ml)			Recovery Rate (%)	
	Original Urine	Twofold Urine	Threefold Urine	Twofold	Threefold
1	428	210	135	98.1	94.6
2	539	261	183	96.8	101.9
3	517	259	174	100.2	101.0
4	433	219	139	101.2	96.3
5	556	281	187	101.1	100.9
6	644	314	218	97.5	101.6
7	826	409	279	99.0	101.3
			mean ± S.D.	99.1 ± 1.8	99.7 ± 2.9

Results

The standard curve for citrate was obtained by injecting standard citrate solutions, in concentrations ranging from 200 μg/ml to 800 μg/ml, into the HPLC column with an auto injector and measuring the height of each peak on conductivity chromatograms (sensitivity range, 0.05 AUFS) (Fig. 3).

Table 1 shows the results of urinary citrate in ten samples from three assay runs. The coefficient of variance for these samples was 1.7–6.2%. Citrate (Wakoh-junyaku Japan) was added at 200 μg or 400 μg per sample to seven

Table 4. Urinary citrate excretion values in control subjects and stone formers

	Male (M)	Female		*P*-value		
		Follicular phase (F)	Luteal phase (L)	M vs F	M vs L	F vs L
Control	423.6 ± 163.7	428.5 ± 173.5	488.5 ± 203.5	NS	$P<0.01$	$P<0.01$
Stone patient	310.9 ± 188.5	345.4 ± 168.4	435.4 ± 186.9	NS	$P<0.01$	$P<0.01$
P-value	$P<0.01$	$P<0.01$	NS		mg/day (mean ± SD)	

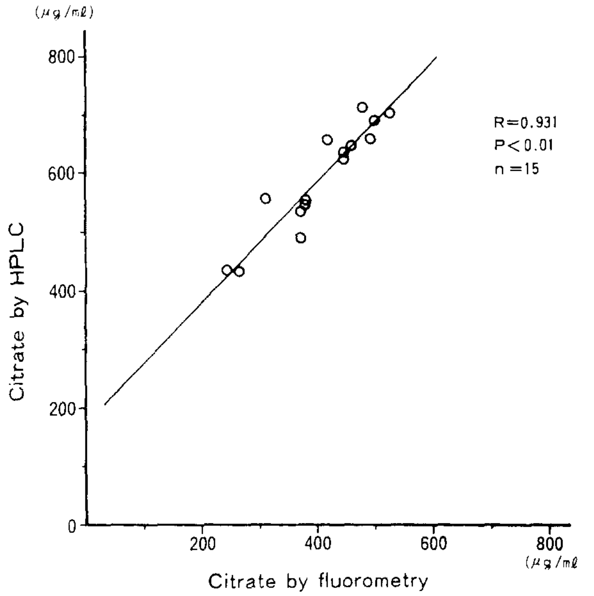

Fig. 4. Correlation between the values high-pressure liquid chromatography (HPLC) and by the fluorometric method

different urine samples. When 200 μg of citrate was added, it was recovered with a yield of 91.0–109.5%. When 400 μg was added, the recovery yield was 92.8–101.3% (Table 2). A dilution recovery rate showed 96.8–101.2% at twofold and 94.6–101.9% at threefold (Table 3). Fifteen samples were assayed for urinary citrate using the conventional fluorometric method and our HPLC method. Good correlations between the results determined, with these two methods were obtained, with a correlation coefficient of 0.931 (Fig. 4). Urinary citrate excretion values for the control subjects and for stone formers are shown in Table 4. There was no significant difference in the excretion value between the male and female control subjects (follicular phase). Urinary citrate excretion values in female controls (luteal phase) was significantly higher than these in both male and female controls (follicular phase) respectively (both groups: $P < 0.01$). In both the male and the female group (follicular phase), stone formers had significantly lower excretion than did control subjects (both groups: $P < 0.01$). There was no significant difference between the control group and the stone group in female stone formers (luteal phase).

Discussion

Until recently we had used the fluorometric method for assay of urinary citrate. This method, however ist troublesome and a great deal of time is required to measure a sample. We have developed a simpler measurement procedure which uses HPLC. This new method provides a quick assay, requiring only 60 min per sample, and it is far simpler than conventional methods. In addition, the use of an autoinjector makes a 24-h continuous assay possible. The coefficient of variance for results obtained using this procedure was low in value (1.7–6.2%). Recovery rate were 91.0–109.5% and 92.8–101.3% after 200 μg and 400 μg additions of citrate respectively. The dilution recovery rates were 96.8–101.2% and 94.6–101.9% at twofold and threefold, respectively. These results indicate that the present assay method is reliable.

The urinary citrate excretion values of calcium-containing stone formers and of control subjects were compared. Citrate excretion values obtained for male stone formers and female stone formers in follicular phase were significantly lower than those for control groups, respectively. It has also been reported that calcium stone formers have low values for urinary citrate excretion [1]. These suggest that subnormal urinary citrate may inhibit urinary stone formation. The excretion of urinary citrate in women during the luteal phase was higher than that of the other two groups. It suggests that progesterone is strongly related to be urinary citrate excretion. The measurement of urinary citrate excretion values is useful to research the cause of formation of calcium-containing calculi and to evaluate the effectiveness of alkali citrate prevention therapy.

Since the technique described in this paper does not require preliminary preparations, complicated treatments and a long determination time, it will probably be of use in routine clinical examinations.

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