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Short communication

Simultaneous determination of oxalic and citric acids in urine by high-performance liquid chromatography

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Abstract

A simple method for the simultaneous determination of oxalic and citric acids has been developed using reversed-phase HPLC. An aqueous solution containing potassium dihydrogenphosphate (0.25%) and tetrabutylammonium hydrogensulphate (2.5 mmol) at pH 2.0 was used as mobile phase. Under these conditions both the components were well resolved and detected at 210 nm. The recovery for oxalic and citric acids was 97% and 102%, respectively. The method presented here was applied to urine specimens of a large number of urolithic patients and control subjects. Because of the simplicity of the method its application provides better means of monitoring the concentration of oxalic and citric acids in the formation of renal calculi.

Keywords: Oxalic acid; Citric acid

1. Introduction

Numerous reports on the analysis of urinary stones confirm calcium oxalate to be the most common constituent of kidney stones. The mechanism by which the initiation of calcium oxalate stone occurs is not fully understood. Crystallization which represents the first phase of urinary stone formation (urolithiasis), depends not only on the state of urinary calcium oxalate saturation, but also on various factors such as the concentration of inhibitors and promoters (specific heterogenous nucleants) of urinary crystallization. The promoting role of high urinary oxalate excretion (hyperoxaluria), even of a mild degree, and the inhibitory effect of citrate on the formation of calcium oxalate stones is well established by both experimental and clinical studies [1–5]. Determination of levels of oxalate and citrate in urines of stone formers (urolithic patients) thus becomes crucial in the investigation of calcium oxalate urolithiasis. Several spectrophotometric and chromatographic methods for the determination of citrate [3,6,7] and oxalate levels [3,4,8–10] in urine are reported in the literature. Chromatographic methods reported so far, are often complex and require large sample volumes. Published HPLC methods for oxalate and citrate are largely limited to oxalate determination, require extensive sample preparation and derivatization [11] or specialized ion-chromatographic methods [12], and are accom-

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panied by endogenous ascorbate interference. In the present work, a simple, rapid and reliable HPLC method for the simultaneous determination of oxalic acid and citric acid levels in urine is developed and applied for the measurement of these constituents in 24-h urine samples of urolithic patients and control subjects.

2. Experimental

2.1. Equipment

The HPLC system consisted of a dual piston HPLC pump Model 6000 (Hitachi, Tokyo, Japan), equipped with a UV detector, a Chromato-pack integrator Model D-2500 Hitachi and a LiChrosorb RP18 column, 5 μm, 250 × 4 mm I.D.

2.2. Chemicals and materials

All the chemicals and reagents used were of analytical grade obtained from E. Merck (Darmstadt, Germany) and were used without any further purification.

The composition of the mobile phase was as follows: 0.25% potassium dihydrogenphosphate and 0.0025 *M* tetrabutylammonium hydrogensulphate buffered at pH 2.00 with orthophosphoric acid. The mobile phase was sonicated and filtered through 0.45 μm acrylic disc filter before use. Aqueous oxalic acid and citric acid standards were prepared in the range of 0.0–4.0 mmol/l and 0.0–16 mmol/l, respectively. These solutions were stable for three months at 4°C.

Twenty-four hour urine specimens were collected from urolithic patients and age-matched control subjects into polyethylene bottles containing 10.0 ml of 6 *M* hydrochloric acid as preservative. Deproteinization of the samples was carried out at ambient temperature by well-mixing of a homogeneous urine sample (1.0 ml) from each collection with 50 mg crystalline sulfosalicylic acid and after 10 min filtering the mixture through a 0.45 μm acrylic disc.

2.3. Chromatographic conditions

The analytical column was cleaned with analytical-grade methanol and equilibrated by pumping the mobile phase to waste at 1.0 ml/min for at least 2 h. Aqueous standard samples for calibration were injected into the liquid chromatograph via a 20-μl loop in a Rheodyne valve at ambient temperature. Subsequently, deproteinized urine samples were analysed under the same conditions. The detector wavelength was fixed at 210 nm. Total analysis time was 10 min.

3. Results and discussion

Typical chromatograms of a standard mixture of oxalic and citric acids and of a deproteinized urine sample are presented in Fig. 1. The concentration of the two metabolites was measured eight times on one day and once daily for six other days. Intra- and inter-assay C.V.s for oxalic acid were 1.3 and 4.0%, and for citric acid 1.2 and 0.2%, respectively. Recovery studies as assessed by the analysis of pooled urine sample showed a recovery of 97% in the case of oxalic acid and 102% in the case of citric acid. Interference studies with urate, glucose, ascorbate and succinate in the concentration of 100 μmol/l each, added separately to a deproteinized urine sample, showed no analytical interference from any of these compounds. The calibration curve for oxalic acid was linear in the range 0.0–4.0 μmol/ml ($y = 12528.41x - 641.943$; $r = 0.999$). The calibration curve for citric acid was linear in the range 0.0–16.0 μmol/ml ($y = 1607.477x - 13.8$; $r = 0.999$). Both peak areas and retention times showed good reproducibilities with C.V. values ($n = 5$) of 0.218 and 0.926, respectively. The detection limit measured as twice the background noise was 8.0 nmol/ml corresponding to 0.16 nmol per injection for oxalate, and was 40 nmol/ml corresponding to 0.8 nmol per injection for citrate. The minimum urinary concentration that could be detected by using the present analytical conditions was 0.2 μmol/ml for oxalic acid and 1.0 μmol/ml for citric acid. The levels

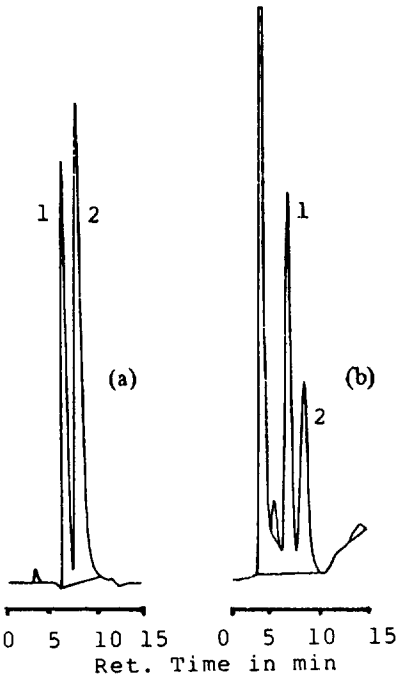

Fig. 1. Typical chromatograms showing (a) standard solution containing 1, citric acid and 2, oxalic acid, and (b) deproteinized urine sample. HPLC conditions: column, LiChrosorb RP18; 5 μm 250×4 mm I.D.; mobile phase, potassium dihydrogenphosphate (0.25%) and tetrabutyl ammonium hydrogensulphate (2.5 mmol) at pH 2.0; flow-rate 1.0 ml/min; detector wavelength 210 nm; injection volume 20 μl.

of oxalate and citrate in 24-h urine samples of control subjects ($n = 14$, mean age 31.21 ± 11.69 years) and of stone patients ($n = 17$, mean age 33.94 ± 17.04 years) are given in Tables 1 and 2, respectively, and the comparison of the mean values is shown in Fig. 2. Urinary excretion of citrate was significantly lower ($P < 0.001$) and that of oxalate higher ($P < 0.01$) in stone patients than in control subjects.

Our particular interest concerns the development of a simple HPLC procedure for the simultaneous determination of citrate and oxalate levels in urine. The present method has the advantage of measuring oxalate and citrate in a single run, thus simplifying a diagnostic procedure for biochemical investigation and follow-up studies of patients with calcium oxalate urolithiasis. In contrast to a recently reported ion-chromatographic method for simultaneous determination of these constituents along with phosphate and sulphate in urine [13], the present method is simple, rapid and requires only deproteinized urine with no extensive extraction steps or dedicated instrumentation.

Two problems normally encountered during the analysis of 24-h urine samples are: (a) the precipitation of calcium oxalate owing to its low solubility in urine; and (b) the shorter retention time of oxalic acid, on the reversed-phase column, due to its hydrophilic nature [14,15]. The first problem was overcome by lowering the pH of the sample to 2.0 with 6 *M* HCl, while the second problem was solved by the addition of tetrabutylammonium hydrogensulphate to the mobile phase. This has considerably enhanced the retention time of oxalic acid. Citric acid did not cause any such problem as it was well resolved during chromatography.

Preconditioning the column with the organic modifier is also necessary after several injections when the retention time of the two acids decreases or unacceptable peak broadening occurs. Methanol was used as organic modifier for preconditioning the reversed-phase column after every ten injections. This helped maintain the reproducibility of retention times and peak areas.

Although derivatization of citrate and oxalate seemed to be essential in some HPLC methods [11,16,17], it was found to result in loss of sample and hence could be a source of error in the quantitation of these metabolites. The levels of oxalate and citrate, found in urolithic patients and control subjects are in agreement with the values reported for these constituents in the recent literature [8,12,17]. It was noted that the citrate excretions were consistently lower, whereas oxalate excretions were higher in urolithic patients than in control subjects. This suggests that the low urinary citrate level may be a serious risk factor in calcium oxalate stone formation. This is because citrate, in addition to its direct inhibitory influence on both the crystallization and crystal growth of calcium oxalate, is very powerful in complexing calcium ions resulting in a decreased ion-activity product for calcium oxalate [18,19]. The differences observed in the urinary oxalate for control subjects and stone

Table 1
Urinary oxalate and citrate levels in control subjects

No.	Age (years)	Urine volume (ml/24 h)	Oxalate (mmol/24 h)	Citrate (mmol/24 h)
1	8	800	0.1078	1.8421
2	24	612	0.1515	1.7512
3	40	430	0.1856	2.3651
4	35	917	0.1983	1.9525
5	52	2246	0.1112	2.1257
6	28	638	0.1324	1.5189
7	36	2175	0.1752	1.7916
8	40	567	0.2205	2.6221
9	37	2410	0.1372	2.3355
10	27	2792	0.1681	2.0414
11	42	4209	0.1554	1.8354
12	33	2850	0.1895	1.8035
13	26	2883	0.1467	1.3962
14	9	428	0.2197	1.5313
Mean	31.2142	1711.2142	0.1642	1.9223
S.D.	11.69	1179.74	0.03	0.339

formers could be attributed to the differences in dietary habits of the two populations.

We conclude that simultaneous analysis on the same urine sample tends to remove possible errors encountered in the separate analyses of these metabolites. This single method of analysis

Table 2
Urinary oxalate and citrate levels in urolithic patients

No.	Age (years)	Urine volume (ml/24 h)	Oxalate (mmol/24 h)	Citrate (mmol/24 h)
1	50	1300	0.2078	0.8565
2	20	980	0.1277	1.4185
3	32	1110	0.3442	1.0036
4	28	2200	0.3712	0.8865
5	58	905	0.2538	0.7871
6	46	832	0.2355	1.2611
7	3	200	0.1356	0.4297
8	31	2320	0.1824	1.4571
9	52	1315	0.2102	0.6186
10	18	2557	0.3045	0.4654
11	18	1930	0.1507	1.1468
12	6	350	0.1927	0.2951
13	55	1223	0.2205	0.4192
14	33	517	0.3331	0.9217
15	47	1386	0.2617	1.6465
16	25	1473	0.2006	0.8751
17	55	1506	0.2301	0.9851
Mean	33.941	1306.11	0.233	0.9102
S.D.	17.038	660.355	0.069	0.379

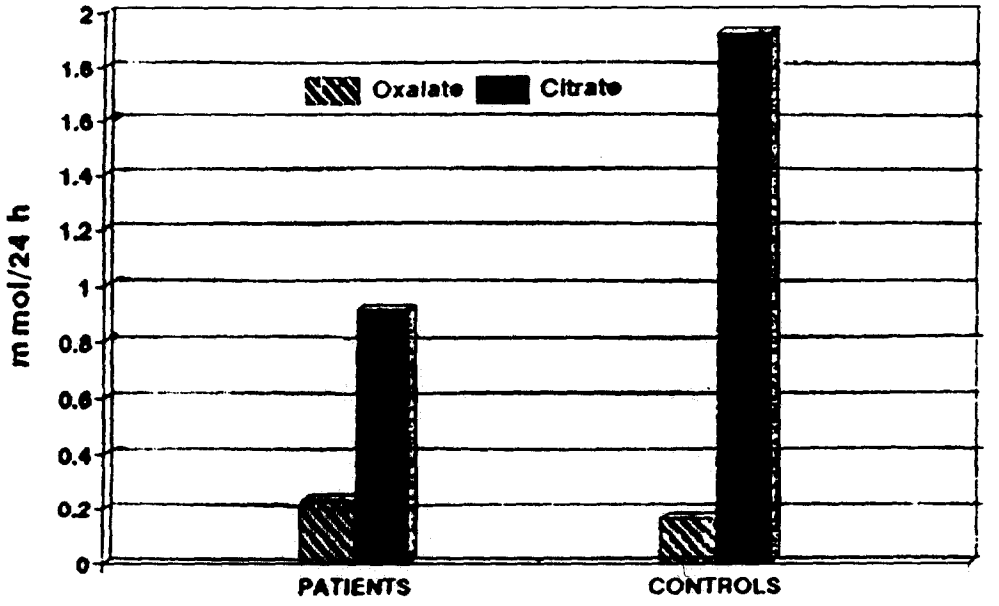

Fig. 2. Mean urinary oxalate and citrate levels in control subjects and urolithic patients.

offers an effective means for biochemical investigation and follow up of patients with calcium oxalate urolithiasis.

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