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Note

Simultaneous determination of citrate and D-isocitrate in urine by isocratic ion chromatography

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Anions capable of forming stable ion pairs with urinary calcium stabilize the urine against the formation of calcium salts by lowering the thermodynamic driving force for crystallization It has been shown that citrate forms such complexes and that a significant fraction of urinary calcium is in the form of these ion pairs [1,2] Another tricarboxylic acid of the Krebs cycle, D-isocitric acid, has also been found to form stable complexes with the calcium ion [3] Since these ion pairs are soluble, the anions have been reported to catalyze the dissolution of calcium oxalate and phosphate phases in vitro by behaving as sequestering agents [4] Moreover, citrate and isocitrate have been found to inhibit the crystal growth of hydroxyapatite through adsorption onto the mineral surface [5] In light of these findings, the urinary concentrations of citrate and D-isocitrate may have important implications in renal stone formation, necessitating the development of a method for their rapid determination

Several methods have been reported for the analysis of urinary citrate To our knowledge, the only method for the determination of D-isocitrate has been reported by Sutor and Percival [6], utilizing enzymatic conversion by isocitrate dehydrogenase However, none of the previously reported methods can be used for the simultaneous analysis of these anions In the present paper, we report on the use of the Dionex QIC analyzer for the determination of D-isocitrate in human urine, simultaneously with citrate The total analysis time of 11 min is considerably shorter than those of previously reported methods

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EXPERIMENTAL

Instrumentation and chromatographic conditions

A Dionex guard column (HPIC-AG3) was used for the analysis with slightly modified conditions from those previously reported by this laboratory for citrate analysis [7] in order to avoid lengthy retention times for the two anions.

Sample and standard preparation

First morning voided urines were collected from eighteen non-stone-forming (NSF) subjects (ten males, eight females) and seventeen known, stone-forming (SF) males The samples were filtered (0 22-μm Millipore filter, Millipore, Bedford, MA, U S A.), acidified by the addition of concentrated hydrochloric acid (1%, v/v) and frozen until use Just prior to analysis, the samples were thawed and, typically, 50-fold dilutions (2%, v/v, in urine) yielded suitable samples for quantitative chromatograms

Calibration standards of citrate (8.0–80 μM) and isocitrate (20–100 μM) were prepared fresh daily from reagent-grade chemicals of their respective sodium salts (citric acid C 7254 trisodium salt dihydrate, D_sL_s-isocitric acid I 1252 trisodium salt, Sigma) and triple-distilled, deionized water

Statistical analysis was performed using the Student's t-distribution and the completely randomized design. A p value of <0 01 was considered significant

RESULTS AND DISCUSSION

Fig 1 shows a typical chromatogram of the citrate and isocitrate anion peaks eluting at 6 6 and 10.3 min, respectively. On the bases of retention time, changes of peak position upon alteration of the eluent solution composition and the spiking of samples with standard solution, the peak at 10 3 min in chromatograms of NSF and SF urine samples was assigned to D-isocitrate Baseline separation was achieved using the chromatographic conditions listed in Table I Linear plots of peak height against analyte concentration, x, were obtained up to 200 μM citrate and 250 μM isocitrate The regression equations describing these plots were found to be y (cm) $= 3\,01 \cdot 10^4 x$ (M) $+ 1\,09 \cdot 10^{-1}$ with a coefficient of correlation (r) equal to 0 9998 for citrate, and for D-isocitrate y (cm) $= 2\,39 \cdot 10^4 x$ (M) $+ 1\,03 \cdot 10^{-1}$ with $r = 0$ 9998

The precision of the method was tested using an SF urine sample found to contain very low levels of both citrate and isocitrate Four aliquots of a 10% (v/v) urine were prepared and assayed thrice The citrate concentration (mean $\pm$ S D) was found to be $24.9 \pm 2\,4$ μM with a coefficient of variation (C V.) of 9 6%, while the average level for D-isocitrate was determined to be $3\,1 \pm 0\,3$ μM (C V $= 9$ 6%). Recoveries of isocitrate (5–10 μM) to several NSF and SF urines, as shown in Table II, were satisfactory, yielding an average recovery of 96.8% ($n = 14$)

Table III shows the comparison of citrate concentration in urine samples from the seventeen NSF subjects as determined by citrate lyase and the ion chromatographic methods A plot of citrate concentrations by the colorimetric

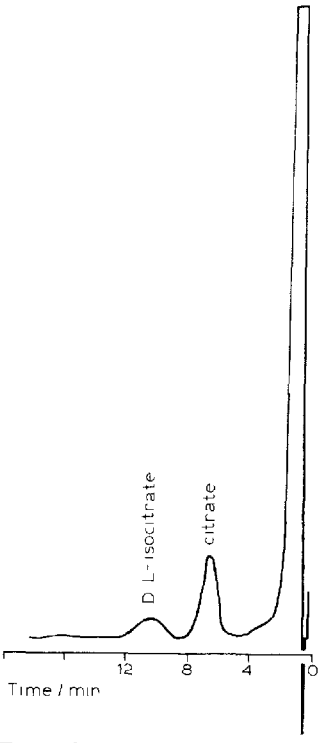

Fig 1 Chromatogram of a 2% NSF urine containing 3 94 m*M* citrate and 1 72 m*M* D-isocitrate Range = 3, chart speed 5 mm/min

TABLE I

CHROMATOGRAPHIC CONDITIONS FOR CITRATE AND D-ISOCITRATE ANALYSIS

Eluent	35 m*M* sodium carbonate
Regenerant	100 m*M* sulfuric acid
Flow-rate	2 0 ml/min
Column	Dionex anion guard column (HPIC-AG3)
Suppressor	Dionex anion micromembrane suppressor
Detector	Dionex conductivity detector
Detector sensitivity	3 μS/cm
Recorder	Omniscribe recorder (Houston Instruments, Graphics Division of Bausch and Lomb, Austin, TX, U S A) 1 V full scale sensitivity

method against those obtained by ion-chromatography (IC) yields a linear relationship which follows the regression equation: y (colorimetric/m*M*) = 1 00(IC/m*M*) − 0 0131 with $r = 0.996$

The method, used to analyze citrate and D-isocitrate in the urine samples from male SF patients and male and female NSF subjects, yielded the results listed in Table IV The citrate concentration of normal male subjects was slightly lower than the value of 2 29 m*M* reported by Menon and Mehle [8] for 24-h

TABLE II

URINARY D-ISOCITRATE ANALYSIS WITH STANDARD D,L-ISOCITRATE ADDITION

Sample[a]	Urine isocitrate (m*M*)	Added isocitrate (μM)	Analyzed isocitrate (m*M*)	Recovery (%)
9	1 08	5 0	1 62	102 5
3	0 51	5 0	1 07	105 9
6	0 60	5 0	1 17	106 4
11	0 22	5 0	0 73	101 4
18	1 43	5 0	1 87	96 9
19	0 22	5 0	0 73	101 4
16	0 16	5 0	0 67	101 5
JS	3 99	10 0	4 82	96 6
JZ	1 17	10 0	1 84	84 8
MAS	2 06	10 0	2 82	92 2
SAS	2 15	10 0	3 07	97 5
JB	0 11	10 0	1 11	100 0
MLR	2 61	10 0	3 01	83 4
AAC	2 76	10 0	3 19	84 8

[a]Arabic numerals denote SF samples, initials denote NSF samples

TABLE III

COMPARISON OF CITRATE IN SEVENTEEN NSF URINES AS DETERMINED BY CITRATE LYASE AND ION CHROMATOGRAPHY

Citrate lyase (y) (m*M*)	Ion chromatography (x) (m*M*)
5 37	5 33
1 47	1 40
2 29	2 30
4 10	4 13
2 06	2 10
2 27	2 23
1 05	1 07
2 10	2 13
1 44	1 53
2 27	2 31
1 38	1 29
1 33	1 27
2 09	2 42
3 54	3 45
2 46	2 50
2 13	2 03
1 60	1 65

urine samples However, the average concentration obtained for SF males is comparable to their reported patient value (1.55 ± 0.13 m*M*). As has been previously noted by other researchers [9], the urinary citrate concentration for

TABLE IV

CITRATE CONCENTRATION IN NSF AND SF URINES

Subject type	Number of subjects	Concentration (m*M*)	Range (m*M*)
NSF, male	10	1 8±0 6	0 4-2 6
NSF, female	8	3 7±2 2	1 2-6 6
SF, male	17	1 3±0 1	0 3-4 1

TABLE V

D-ISOCITRATE CONCENTRATIONS IN NSF AND SF URINES

Subject type	Number of subjects	Concentration (m*M*)	Range (m*M*)
NSF, male	9	0 6±0 3	0 26-1 34
NSF, female	8	0 8±0 5	0 23-1 20
SF, male	16	0 5±0 2	0 27-0 80

female subjects is significantly higher than for males and is corroborated by the results of this study ($p=0$ 0086, Student's t-distribution)

Table V lists the values obtained for the D-isocitrate concentration in both NSF and SF urines Statistical analysis of the three groups in terms of average isocitrate concentration revealed no significant difference among them ($p=0$ 5552, F statistics of the completely randomized design)

CONCLUSIONS

Although the data obtained for SF and NSF urines failed to demonstrate a statistically significant difference between the groups in terms of D-isocitrate level, the ion chromatographic method described for the simultaneous determination of citrate and isocitrate is demonstrated to be a useful analytical tool for the very rapid and reproducible quantification of these anionic species The method may be readily adaptable for use with other biological matrices and/or with pure solutions supersaturated with respect to calcium-containing salts, in which the ions may be introduced as dissolution catalysts or crystallization inhibitors

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