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ISOTACHOPHORETIC DETERMINATION OF URINARY CITRATE IN NATIVE URINE

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SUMMARY

The technique of isotachophoresis has been used to develop a specific and sensitive method for the determination of citrate in unprocessed urine. The specificity of the isotachophoretic method was assessed using citrate lyase which caused disappearance of the isotachophoretic citrate signal. The isotachophoretic method compared favourably with the enzymatic method (citrate lyase) for urinary citrate. The normal range for urinary citrate in 25 healthy individuals, as found by isotachophoresis, was 0.33—2.89 mmol/24 h with a mean of 2.1 mmol/24 h.

INTRODUCTION

The determination of urinary citrate is of interest in urolithiasis research because of the known ability of citrate to form soluble complexes with calcium [1]. Consequently, the calculation of the activity products of the ions involved in stone formation [2] requires knowledge of the citrate concentration in urine. Currently, the determination of citrate in biological fluids can be performed using the pentabromoacetone method [3] or the enzymatic method [4] using citrate lyase (E.C. 4.1.3.6.) (CL).

Isotachophoresis has been successfully applied to the determination of mandelic acid, phenylglyoxylic acid, hippuric acid and purines or pyrimidines in urine [5, 6]. This promising method has not been systematically applied to the determination of other naturally occurring urinary constituents. The present investigation describes the conditions under which urinary citrate can be quantitated by isotachophoresis.

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MATERIALS AND METHODS

Urine samples

Urine (24-h sample) was collected from 25 healthy individuals (laboratory staff), 10 male, 15 female, who were under their usual self selected diet and had no known renal or other diseases. Urine was collected without additives in plastic bottles and kept at 4°C during the collection period.

Measurement of urinary citrate by citrate lyase

The estimation of urinary citrate was performed by using the commercially available test kit of Boehringer (Mannheim, G.F.R.) using Welshman and McCambridge's method [7]. In the experiments designed for complete degradation of citrate in urines, the final concentration of the enzyme was 1 U/ml at a pH of 7.5 in 50 mmol imidazol—HCl buffer with an incubation time of 10 min.

Measurement of urinary citrate by isotachophoresis

The isotachophoretic analyses were performed in an LKB 2127 Tachophor (LKB, Bromma, Sweden) equipped with a 23 cm × 0.5 mm I.D. capillary. The starting voltage was 2 kV with a final voltage varying between 4 and 12 kV at 150 μA and 12°C. UV absorption at 254 nm was used for quantitation. The thermosignal could be used reliably for identification of the citrate. The time of analysis depended on the electrolyte content of the sample and varied between 20 and 60 min.

The chemicals used were commercially available and of analytical grade. The water used was distilled twice. In addition, the purification procedure for the terminating electrolyte solution was performed, as proposed by Everaerts et al. [8]. Leading electrolyte: $5 \cdot 10^{-3}$ *M* hydrochloric acid Suprapur (E. Merck, Darmstadt, G.F.R.); $1 \cdot 10^{-3}$ *M* sodium chloride (p.a. grade, E. Merck); 0.4% hydroxypropylmethylcellulose (Methocel 90 HG, 15,000 cps, Dow Chemicals, Midland, MI, U.S.A.). No adjustment of the pH (2.2) was carried out.

Terminating electrolyte: acetic acid, $5 \cdot 10^{-3}$ *M* (p.a. grade, E. Merck). Injection: the samples were injected through a septumless syringe injector as in Fredriksson's method [9] with an AA 701SN 10-μl Hamilton microsyringe equipped with a platin needle and tungsten plunger. The volume of injection varied in the range 2—10 μl. Citrate was quantitated by measuring the zone length with a graticula or a ruler. The chart speed was 0.5 or 1 mm/sec.

RESULTS

Measurement of citrate in aqueous solutions and urines

Aqueous solutions of citrate were tested over a range of 0.05—13 mmol/l. The injected volume was adjusted appropriately so that 5—10 nmol of citrate were injected. In aqueous solutions, the citrate signal, i.e. a non-UV-absorbing zone, could be easily identified by the specific stepheight of the thermal signal. It was preceded and followed by UV-positive zones (impurities?). Similarly, in urine, the citrate signal was sandwiched between even more pronounced UV-

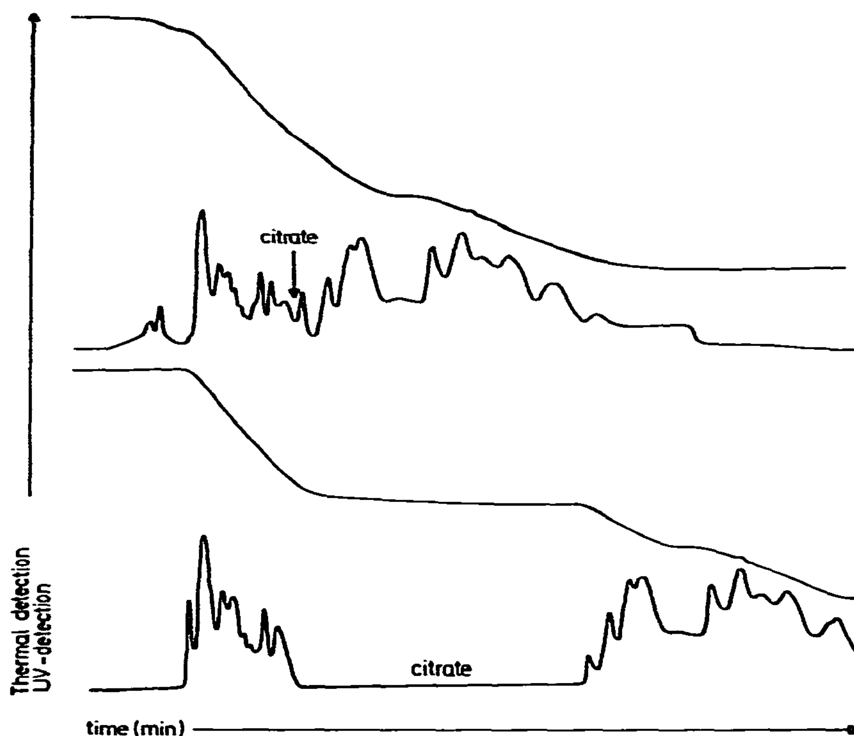

Fig. 1. Typical isotachopherograms of native urine before (lower two curves) and after (upper two curves) treatment with CL. Citrate can be reliably identified by both the non-UV-absorbing zone and by the specific step height of the thermal signal. After treatment with CL, a small, non-UV-absorbing incisure is detectable, which could correspond to non-converted citrate. Identification or quantitation of this zone is not possible. Injected volume: 10 μl native urine; incubation medium and procedure as given in text. The UV- (and thermal) traces of Figs. 1, 2 and 4 represent the middle parts of the complete isotachopherograms. Phosphate is the UV-negative zone preceding the (unidentified) UV-absorbing zones before the citrate.

absorbing zones (Fig. 1). This was obtained in all the urine samples examined. In some samples of freshly voided urine, significant changes with time were observed in the UV-absorbing zones which preceded the citrate zone. The zones preceding citrate diminished while at the same time the citrate zone length increased (Fig. 2). This process ceased after approximately 2.5 h. Thereafter no further changes of the length or shape of the UV-absorbing zones or the citrate zone were observed for up to 16 h. The following experiments therefore were all conducted with urine, which had been allowed to stand at least for 5 h at room temperature.

The calibration curve for standard aqueous solutions as well as the recovery curve of citrate standard added to pooled urine are given in Fig. 3.

The specificity of the isotachophoretic signal for citrate was examined by analysis of its reaction to treatment with CL. In none of the urines examined (n = 47) could a non-UV-absorbing zone, corresponding to citrate, be measured or detected after treatment with CL (Figs. 1, 4). The conditions of CL treat-

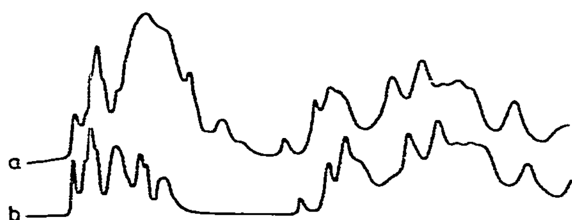

Fig. 2. Isotachopherograms of freshly voided urine. Injections: (a) immediately after voiding and (b) after 60 min standing at room temperature. The significant qualitative and quantitative changes in the zone length of citrate and the UV-absorbing zones preceding citrate suggest dissolution of citrate-containing complexes. Injected volume: 8 µl, procedure as in Fig. 1.

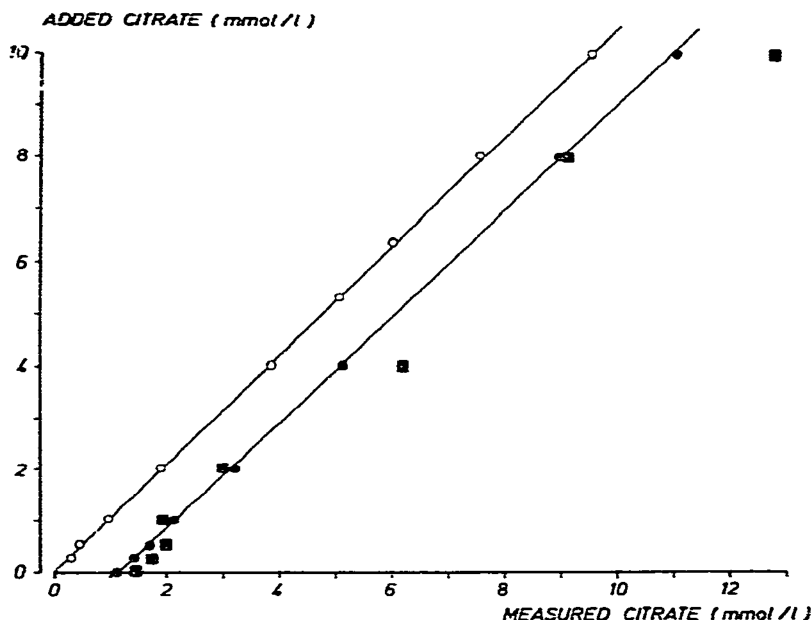

Fig. 3. Calibration curves: (○) citrate in aqueous solutions (isotachophoretic method); (●) internal standard (citrate added to pooled urine, isotachophoretic method) and (■) internal standard (enzymatic method). For details see text.

ment were: 1 U/ml; pH 7.5; 50 mmol imidazol—HCl buffer; this medium does not interfere with the isotachophoretic analyses.

The minimum amount of citrate which would be detected and measured under the above conditions is 0.2—0.4 nmol. In aqueous solutions, the conversion of citrate to oxaloacetate by the action of CL could be followed quantitatively, oxaloacetate being recorded as a zone with weak UV-absorption preceding the UV-negative citrate zone. In urine however, no clearcut detection of oxaloacetate was possible.

Recovery and reproducibility

The recovery of 1 mmol/l citrate added to 15 urine samples was 100 ± 2%

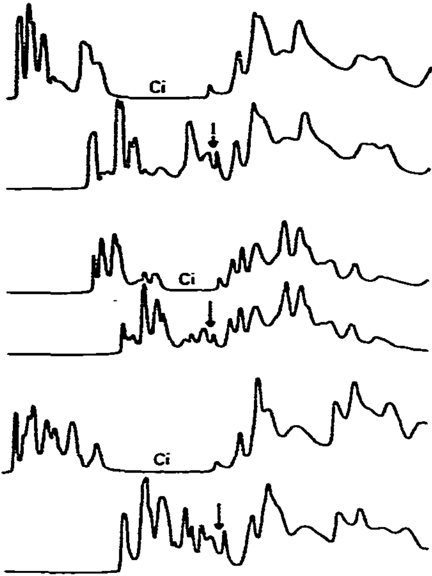

Fig. 4. Isotachopherograms (UV recording) of three urines before and after treatment with CL. Ci: non-UV-absorbing citrate zone. The arrows indicate a small non-UV-absorbing zone, which might correspond to non-converted citrate. Injected volume: 5 μl of native urine, procedure as in Fig. 1.

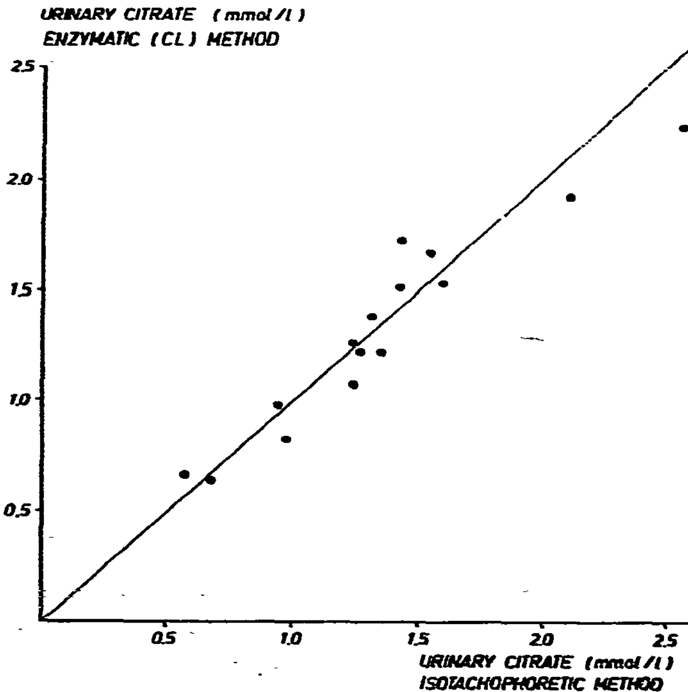

Fig. 5. Plot of 24-h urinary citrate determinations by the isotachophoretic method against the enzymatic method. The straight line indicates the line of identity. The results show a high degree of correlation ($r = 0.997$). For details see text.

(x ± S.D.). The reproducibility in aqueous solutions (10 sequential measurements) was 100 ± 0.7% and 102 ± 1.6% in urine samples.

Effect of storing and freezing

Storing at −25°C for up to one week and repeated freeze thawing did not markedly (± 5%) after the isotachophoretic measurements of citrate in urine.

Comparison of the enzymatic and the isotachophoretic methods for determination of urinary citrate

The citrate content of 24-h urine samples (n = 16) was analysed both by the isotachophoretic and by the enzymatic method. A close correlation (r = 0.997) between the results of the two methods was found (Fig. 5). In addition, the recovery of added citrate in pooled urine (n = 15) was also analysed by both methods (Fig. 3). While the recovery of added citrate analysed by isotachophoresis was virtually 100% and independent of the amount of added citrate, the values obtained with the CL method showed greater deviations from the expected values.

DISCUSSION

Isotachophoresis is based on the principle that the net mobility of each of the participating ions is constant under defined conditions. At equilibrium, therefore, the ion concentration in each unit length of the given zone is constant. Quantitation can then simply be achieved by measuring the length of each sample zone. The present work shows that quantitative determination of urinary citrate is possible by isotachophoresis with high accuracy and precision. The method is applicable to unprocessed urine samples either voided or stored for prolonged periods of time.

The results obtained by isotachophoresis are comparable with those obtained by the enzymatic CL method and show even less variance than the latter method. The values found for the 24-h urinary excretion of citrate with isotachophoresis are in accordance with values reported in the literature for the CL method [7, 10]. The values reported for the pentabromoacetone method are higher [11] — this is presumably due to its lack of specificity. The CL method was originally developed for the estimation of citrate in fruit juices etc. [4]. It has been applied to urine by Welshman and McCambridge [7].

While the isotachophoretic measurement of urinary citrate seems to be more precise than the CL method, isotachophoresis has one draw back, i.e. a relatively long run-time. However, the method offers one distinct advantage, i.e. the possibility of co-determination of other urinary anions (e.g. oxalate, sulfate, glycolic acid, uric acid, orotic acid [8, 12]).

The isotachophoretic method, using the technical details described above, provides a useful investigational tool for examining the role of urinary citrate in renal stone formation [2, 10] or other disease processes [13].

ACKNOWLEDGEMENT

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