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DETERMINATION OF URINARY OXALATE BY ION CHROMATOGRAPHY. SOME MODIFICATIONS

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Ion chromatography was used to determine urinary oxalate concentration. The minimal detectable limit in a standard solution was 0.02 μg/ml, and the regression line for the standard curve from 0.5 to 10 μg/ml had a significant correlation coefficient ($p<0.01$), but it was difficult to obtain reproducible results with ion chromatography. There were two obstacles: Urine was always oversaturated with calcium oxalate, and the presence of sulfate, the peak of which is next to the oxalate peak on the chromatogram of urine, seemed to interfere with oxalate conductivity. Comparison of 4 different dilutions of urine showed that the oxalate conductivity was highest in the 100-fold dilution and decreased gradually with increasing concentration. The conductivity of the oxalate standard (1 μg/ml) also decreased as the concentration of sulfate increased.

Therefore, it was concluded that to obtain reproducible results urine should be diluted 100-fold and that a recovery test by adding 0.25 μg/ml oxalic acid should be performed on all urine samples.

Key words: Urinary oxalate, Ion chromatography, Recovery test

INTRODUCTION

The role of urinary oxalate in calcium oxalate stone-formation is hardly questionable. A variety of methods have been developed for oxalate determination, and recently, enzymatic methods are gathering attention because of their specificity. The radioenzymatic isotope dilution method, an enzymatic method, was devised by D. J. Bennett and his associates (1978)[1]. This technique was simplified by one of us[2], who with collaborators, also measured the oxalate in urine, plasma, and common Japanese foods[3~5]. The radioenzymatic method which was first demonstrated as being more simple, sensitive, and reproducible than earlier methods, has not, however, become popular among oxalate researchers because its procedure is too sophisticated, requiring considerable technical know-how and because isotopes are used. Later, a method using high-performance liquid chromatography (HPLC) with electrochemical detection was used for the determination of oxalates in the urine by W. J. Mayer and his associates (1979)[6]. But this method is limited in that the urinary oxalate has to be precipitated and extracted and no suitable internal standard is known. Several modifications[7~9] have been attempted, but determination of urinary oxalates with HPLC is in need of improvement.

On the other hand, ion chromatography, which Small and his associates applied in 1975[10], has now been perfected[11]. This technique which utilizes ion-exchange separations, coupled with a unique detector composed of a background suppressor and an electrical conductivity cell is now a populular method of separating and quantifying ions (both organic and inorganic). It was first used by Mahle and Menon (1982)[12] to measure the oxalate in unprocessed urine. We also used a

slightly modified version of ion chromatography, and measured the oxalates in urine. The recovery rate of oxalate added to urine which had been diluted 10-fold was found to be unexpectedly low. Recently Menon and Mahle[13] modified their procedure and reported that the mean recovery of oxalate added to urine which had been diluted 100-fold was 101 % and concluded that the urine matrix at that concentration did not interfere with oxalate recovery. The present paper describes some improvements and modifications in determining urinary oxalates by ion chromatography.

MATERIALS AND METHODS

Special-grade chemicals were obtained from Wako Pure Chemicals, Ltd. Oxalate decarboxylase (Sigma Chemicals Co.) had a specific activity of 2.68 Unit/mg Protein (lot 119 C-6820). The ion chromatograph used was a Model IC 100 Ion Chromatoanalyzer, available from Yokogawa-Hokushin Electric Works. As eluents, 0.004 M $NaHCO_3$ and 0.004 M Na_2CO_3 were used at a flow rate of 2 ml/min. All the solutions were reconstituted in deionized, triply distilled water and were stored in plastic containers. Standard solutions were freshly made before each use. The precolumn (4.6ϕ50 mm) was filled with styrenedivinyl benzene beads with a surface agglomerated with anion latex particles (Precolumn AX-1). The analytical column (4.6ϕ250 mm) was filled with the same anion-exchange resin (Separator AX-1). The suppressor was made of an ion-exchange membrane tube (Suppressor CX-1). The scavenger used was 0.05 M dodecylbenzenesulfonic acid at a flow rate of 2 ml/min. All the columns, the suppressor, and the detector were kept at 40°C in a thermostable chamber. The conductivity of the anions was measured by means of a unique conductometric detector against the background of carbonic acid.

The urine was collected in a plastic bottle and was acidified to pH 1.0 with concentrated HCl. One-ml aliquots of urine were diluted 10-, 20-, 50-, and 100-fold with deionized distilled water. In order to add 1 μg/ml oxalate to 10-fold urine, for example, 1 ml of urine and 1 ml of 10 μg/ml oxalate were added to an 8-ml aliquot of deinonized distilled water. In this manner, 0.5, 1, 2, and 5 μg/ml of oxalate were added to urine diluted as above. About 500 μl of each sample was injected into a loop injector, which loads 100 μl of the sample into the column. The oxalate peak appeared from 8 to 10 minutes after the injection. As the oxalate concentration is proportional to its conductivity, it was measured as the peak height from a tangential base line. After the oxalate peak, two injections of 0.5 M Na_2CO_3 were followed in order to wash and clean the column, which took 8 minutes to settle in the case of 100-fold diluted urine and longer for 10-fold diluted urine.

RESULTS

Standard solutions of oxalate were injected into the chromatograph, and the conductivity was measured as the peak height. This peak specifically represented oxalate, because the peak increased in height upon the addition of standard oxalate and completely disappeared after the treatment of the sample with oxalate

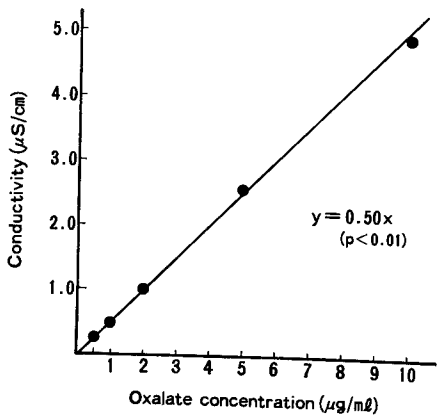

Fig. 1. Standard curve for oxalate. Standard solutions of oxalic acid (0.5 to 10 μg/ml) were injected into the chromatograph and the conductivity was measured as the peak height (sensitivity=3 to 10 μS/cm). The minimal detectable limit was 0.02 μg/ml in a standard solution (sensitivity range= 1 μS/cm).

decarboxylase. In Figure 1, the concentration of oxalate is plotted on the abscissa, and the conductivity, on the ordinate. The line of regression for the standard curve is expressed as $y=0.5x$, with a significant correlation coefficient ($p<0.01$).

In order to determine the recovery rate of oxalate added to urine, increasing quantities of oxalic acid (0.5 to 5 μg/ml) were added to 3 different dilutions of 2 different urine samples, and the differences in conductivity were measured. The recovery of added oxalate in a 10-fold dilution was less than 60%, while that in a 20-fold or 50-fold dilution was 98.1% or 97.7% respectively, as is shown in Figure 2. These results suggested that the 20-fold dilution gave a sufficiently reproducible result. However, this was proved wrong by the following experiment, in which the oxalate conductivity was compared in 4 different dilutions (10-, 20-, 50- and 100-fold) of 3 different urine samples, taking that of the 100-fold dilution as the standard. The conductivity was highest in the 100-fold dilution and decreased gradually with increasing concentration, as is shown in Figure 3.

In order to determine how other ions interfere with oxalate conductivity, the oxalate standard (1 μg/ml) with other anions near the normal urinary level was measured. Chloride, phosphate, nitrate,

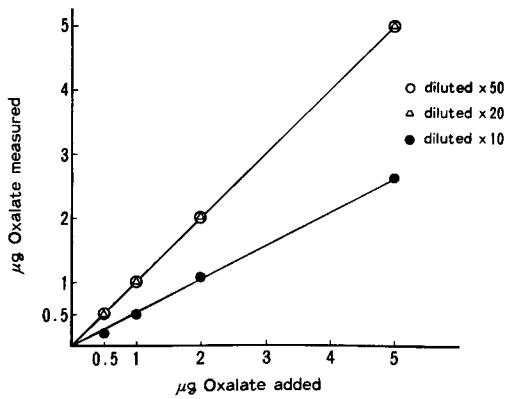

Fig. 2. Recovery of oxalate added to urine. Increasing quantities of oxalic acid (0.5 to 5 μg/ml) were added to urine (dilute ×10, ×20, or ×50) and the differences in conductivity were measured.

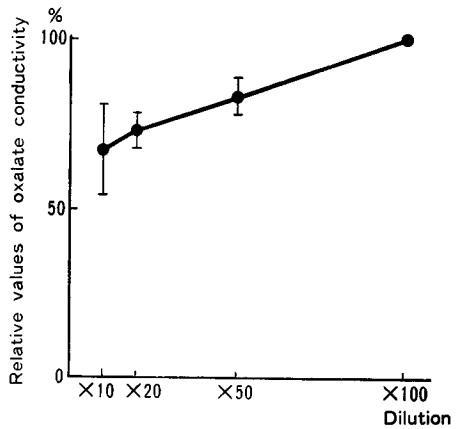

Fig. 3. Relative values of oxalate conductivity of three urine samples as measured by 4 different methods of dilution, using the mean value of the 100-fold dilution as the standard (100%).

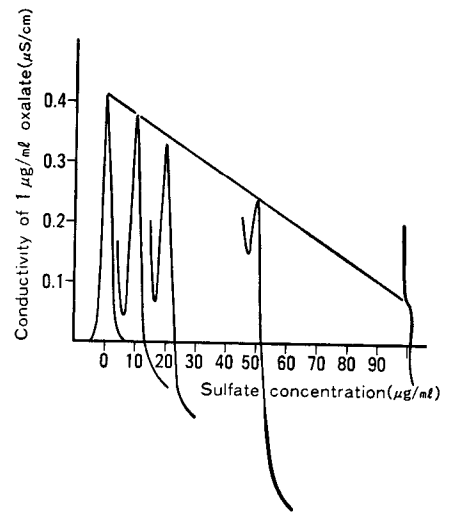

Fig. 4. Effect of sulfate on oxalate conductivity. The same concentration of oxalic acid (1 μg/ml) with increasing concentrations of sulfuric acid (10 to 100 μg/ml) was measured.

citrate, and urate did not interfere with oxalate conductivity, but sulfate did. The results of the measurement of the oxalate standard (1 μg/ml) with increasing concentrations of sulfate are shown in Figure 4. The oxalate peak was shortened as the concentration of sulfate increased.

Urinary oxalate was measured in 10 urine samples diluted 100-fold and with 0.25 μg/ml oxalic acid added. The recovery rate of the added oxalate was 95.8

%. The minimal detectable limit in 100-fold diluted urine was 0.025 μg/ml, and that in a standard solution, 0.02 μg/ml.

DISCUSSION

Recently ion chromatography (IC) has become popular because it is simple and can be used routinely to separate a variety of nonchromophoric ions and allow sensitive detection using a conductivity detector. Special low-capacity ion-exchange resins are used in order to obtain the efficient separation of the ions. To reduce the baseline noise level, the eluent of the first ion-exchange column (the separator) is pumped into the second ion-exchange column (the suppressor), which reduces the usually high background conductivity. IC is now widely accepted because it offers a faster and more accurate method for simultaneously determining many species of ions.

IC was first used by Mahle and Menon to determine the oxalates in urine and was evaluated as simple and precise, with a total impression index of 4.9%[12]. Also, the specificity of the oxalate peak on the chromatogram was confirmed by digesting oxalate with its specific enzyme, and by means of studying radioactivity. However, in our further experience with the assay we encountered the obstacle of oversaturated urine, which contains a variety of substances. Two questions arose: Was a 10-fold dilution weak enough and was the use of a standard oxalate concentration to measure the urinary oxalate concentration appropriate. Earlier findings on urinary oxalate supersaturation demonstrated that a 10-fold dilution of urine is not always weak enough to obtain a subsaturated level in every urine sample saturated[14]. Calcium oxalate crystals form in an alkaline eluent in oversaturated samples and will eventually pass through the column. In the presence of an alkaline eluent, only ionized oxalate can be measured accurately. Quite recently Menon and Mahle modified their technical procedure of urinary oxalate determination by IC[13], probably because they had encountered a similar situation. We reached almost the same conclusion, but the results shown in Figure 3 raised some more questions. An experiment was conducted to look for factors which might interfere with oxalate conductivity. The presence of sulfate was found to interfere with oxalate conductivity and could make it difficult to interpret the oxalate peak, as the sulfate peak is next to the oxalate peak. This was proved by the present experiment. The results shown in Figure 4 indicate that the oxalate peak of the same 1 μg/ml of oxalic acid on the chromatogram is shortened as the concentra tion of sulfate increases. IC has the advantage in that it can simultaneously analyze multiple major ions but has the disadvantage of not enabling analysis of a single minor ion with an unseparable and overlapping major ion on a chromatogram. There is also the possibility that another ion which is next to the oxalate peak may interfere with its conductivity; in such cases, calculation using secondary differentiation may be required to obtain accurate results.

When sulfate interferes with the oxalate conductivity, a recovery test by adding from half to an equal amount of the standard oxalate to 100-fold diluted urine would be one way to obtain reproducible results. In measuring substances in a biological fluid, another problem is column contamination. As the concentration of urine increases, the column is contaminated more. In order to be injected into the IC column, the sample should be diluted as far as the IC can be quantified. A diluted sample saves the column from contamination and also keeps it working longer.

In conclusion, a modification of the procedure in measuring the urinary oxalate by IC is proposed : the urine sample should be diluted 100-fold, and a recovery test should be performed each time by adding 0.25 μg/ml oxalic acid. This recovery test is valuable in eliminating the sulfate peak on the chromatogram of urine.

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和文抄録

イオンクロマトグラフィーによる尿中蓚酸測定

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イオンクロマトグラフィーを尿中蓚酸測定に応用した．標準曲線は蓚酸 0.5～10 μg/ml の範囲で有意の直線関係を示した．測定限界は 0.02 μg/ml であった．しかし尿中蓚酸測定をおこなった結果，再現性を良好にするには2つの問題点を発見した．尿は常に蓚酸カルシウムの過飽和溶液であることと，クロマトグラム上で隣のピークである硫酸が干渉することであった．その証拠に尿を稀釈し蓚酸のピークを測定し求めた値を比較すると10倍稀釈の蓚酸値がもっとも低く，稀釈倍数とともに値は増加し，100倍稀釈の値が最高であった．また，一定量の蓚酸 1 μg/ml を含む溶液中に共存する硫酸濃度が増加すると，その蓚酸の回収率は低下する．

以上より，再現性を良好にするには各尿サンプルの100倍稀釈と，それに蓚酸 0.25 μg/ml を添加したものを測定し，これらにより値を求めることが必要である．