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Conductivometric Determination of Urinary Oxalate with Oxalate Decarboxylase

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Summary. An enzymatic method for determination of urinary oxalate is described: the acidified urine samples are extracted with chloroform. This manipulation improves the blank values considerably. 1 ml of extracted urine is incubated with oxalate decarboxylase. The CO_2 released from the medium is absorbed by $Sr(OH)_2$. The change in conductivity measured in the $Sr(OH)_2$ solution is linearly proportional to the oxalate concentration in urine and the method is specific for oxalate. The mean recovery is 93.2 ± 2.5%. The coefficient of variation calculated from 28 determinations is 12.6%. The detection limit is 35 nmol. 1 ml of urine is usually sufficient for determination. The mean 24 h urine oxalate excretion of 11 healthy men and 16 women was 240 ± 20 μmol.

Key words: Urine, Oxalate, Conductivometry, Oxalate decarboxylase.

Although several methods have been described for the determination of oxalate in urine, this measurement is still a problem in clinical chemistry. The older chemical methods tend to be cumbersome though they may have the advantage of a final colorimetric assay [8]. The techniques more recently described involve gas chromatography [2, 5, 6, 15, 16, 21], high pressure liquid chromatography [13, 14], isotachophoresis [19], or make use of enzymatic degradation [4, 7, 11, 12, 22]. Recently also immobilised enzymes have been applied [1, 10].

In the present study, an enzymatic technique was coupled to conductivity measurement. CO_2 released by decarboxylation of oxalate was absorbed in $Sr(OH)_2$ solution. Oxalate could therefore be assayed by the change in conductivity in the $Sr(OH)_2$ solution, by a modified version of a method previously described [18].

Methods

Enzymes

For the first assays oxalate decarboxylase was extracted from mycelia of Collybia velutipes and precipitated with 33%–50% acetone [20]. The enzyme solution was kept frozen in samll aliquots at −20 °C. The enzyme activity obtained per 90 g mycelia from 7 extractions was 1,157 ± 197 nmol/min (mean ± SEM). When it became available, the enzyme was bought from SIGMA Chemical Co., St. Louis, USA.

Oxalate oxidase was prepared from an extract of barley seedlings heated to 80 °C for 3 min [3].

Determination of Oxalate in Urine

24 h urine specimens and morning urine specimens from healthy adults (laboratory personnel) were collected in plastic bottles containing 1 ml of chloroform and kept at 4 °C until the measurement was done (not longer than 7 days). The day before the assay the urine was acidified to pH 1.6 with conc. HCl to drive out the CO_2 and to prevent oxalate precipitation [9]. The next day the pH was adjusted to pH 3.2 with 2 N NaOH. Urine was then extracted three times with chloroform (1:1 (vol/vol)) and heated to 80 °C for 20 min. After cooling to room temperature, the urine was bubbled for at least 30 min with oxygen, freed from CO_2 by bubbling through a gas wash flask containing 1 N NaOH. Aliquots of 1 ml of urine were pipetted into 10 ml penicillin vials. In these vials an Eppendorf cup without a cap had been inserted. Then, 0.5 ml of glycine buffer (pH 3.2), 5 μCi [^{14}C]oxalate and water to give a final volume of 2.5 ml were added to the urine. The penicillin vials were flushed with a stream of CO_2-free oxygen (0.8 l min^{-1}) during the time the solutions were added, to prevent uptake of CO_2. Then 0.5 ml of $Sr(OH)_2$ solution was added to the Eppendorf cup and 20 μl (10 m-units) of enzyme solution to the incubation medium. The penicillin vial was closed immediately by a rubber stopper, and the latter clamped with an aluminium ring using a "Fermpress". For the blanks the enzyme was omitted. The vials were slightly shaken horizontally at room temperature for 16 h (ν: 55 min^{-1}; amplitude: 0.02 m).

The $Sr(OH)_2$ solution was prepared as follows: 4.5 ml of a saturated solution of $Sr(OH)_2$ were diluted to 250 ml with CO_2-free distilled water. The diluted $Sr(OH)_2$ solution was always prepared fresh on the day of the experiment and kept in a CO_2-free oxygen atmosphere.

After the 16 h incubation the rubber stopper was removed, the $Sr(OH)_2/SrCO_3$ solution in the Eppendorf cup aspirated and diluted with 1 ml of CO_2-free distilled water into a plastic tube.

The conductivity was measured using an electrode EA 240 connected to the conductometer (Konduktoskop, E 365 B, Metrohm AG, Herisau, Switzerland). Values for urine blanks were approximately 200–230 μ Siemens (μS) and sample values in the range of 80–180 μS.

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Table 1. Influence of chloroform extraction on $[^{14}C]HCO_3^-$ concentration

Urine	% Radioactivity		
Treatment	After 1st treatment	After 2nd treatment	After 3rd treatment
Shaking *and* chloroform extraction	11.8/9.9	4.5/3.2	1.3/0.9
Shaking alone	28.1	18.7	17.0

0.75 μCi $[^{14}C]HCO_3^-$ were added to 150 ml urine acidified to pH 3.2. The radioactivity was measured and taken as control value (100%). The urine was divided into three samples of 50 ml. Two samples were shaken three times with chloroform (1:1 (vol:vol)) for 5 min, the third sample was shaken three times without chloroform. The radioactivity remaining in the urine was measured after each treatment and expressed as percentage of the control value

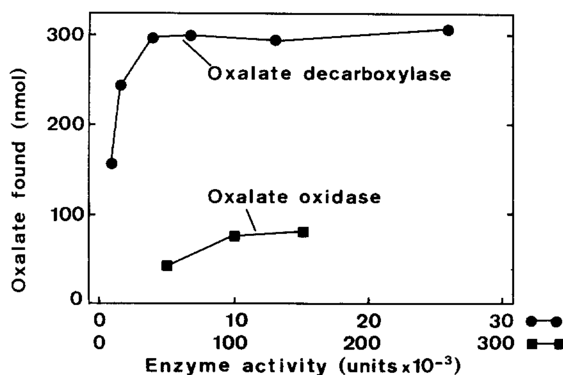

Fig. 1. *Conversion of Oxalate to CO_2 by Different Amounts of Oxalate Decarboxylase and Oxalate Oxidase.* 0.5 ml of urine were incubated with different amounts of oxalate decarboxylase as described under "methods". In the case of oxalate oxidase 0.125 ml of urine were incubated with 8 mM succinate buffer pH 3.5 in a final volume of 2.5 ml for 16 h. The CO_2 formed was measured in the same way. Incubation with oxalate decarboxylase (•—•), with oxalate oxidase (▪—▪)

The standard curve was obtained by incubating 0 to 400 nmol oxalate. The values varied from experiment to experiment from 230–260 μS for the blanks and from 65–95 μS for 400 nmol of oxalate.

For radioactivity measurement, 350 μl of incubation medium was transferred to 10 ml scintillation fluid composed of 750 ml of toluene, 250 ml of triton X-100 and 7 g of butyl-PBD [5-biphenyl-4-yl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] (Ciba Geigy, Basel, Switzerland). Since the oxalate decarboxylase transforms oxalate to CO_2 (absorbed by $Sr(OH)_2$) and to formate (left in the incubation medium), 50% of the total radioactivity added as oxalate should remain in the incubation medium. A value significantly higher than 50% suggested that either the oxalate was not completely transformed or that CO_2 did not diffuse completely out of the medium Repeat analyses were performed on these samples.

Results and Discussion

Extraction with Chloroform

As seen from Table 1, shaking of the urine with chloroform was very efficient in extracting the radioactivity added as $[^{14}C]HCO_3^-$. When, after acidification of the urine, pCO_2 and pH were measured and HCO_3^- concentrations calculated (data not shown), the amount of radioactivity recovered in the urine was higher than what could be expected to be due to dissolved CO_2 and HCO_3^-. This suggests that most of the radioactivity would be present in the form of H_2CO_3, which seems to decay slowly into CO_2 and H_2O. As an apolar compound H_2CO_3 seems to be efficiently extracted by chloroform.

To exclude the possibility that oxalate was extracted from urine with chloroform, $[^{14}C]$oxalate was added to the urine and the radioactivity in the chloroform was measured after the extraction procedure. The values found were not different from the blank, indicating that oxalate was not extracted with chloroform.

Absorption of CO_2 by $Sr(OH)_2$

In order to determine the speed of absorption of CO_2 by the solution of $Sr(OH)_2$, $[^{14}C]NaHCO_3$ was added to the incubation buffer and the radioactivity remaining in the medium as well as the conductivity of the $Sr(OH)_2$ solution measured over time at room temperature. After 10 h all CO_2 was absorbed by the $Sr(OH)_2$ solution and no radioactivity was left in the incubation medium. An incubation time of 16 h was therefore chosen for further experiments.

Choice of the Enzyme

In Fig. 1 the action of the two enzymes oxalate decarboxylase and oxalate oxidase is compared. 4 m units of oxalate decarboxylase were necessary to convert all the oxalate to CO_2 and formate. On the other hand, 100–150 m units of oxidase were required to convert the oxalate in a urine containing approximately 1/4 the concentration of oxalate. Thus, decarboxylase is less inhibited by urine than oxidase. In all subsequent experiments oxalate decarboxylase was chosen and 10 m units of enzyme used. Preliminary precipitation of oxalate to remove inhibitors was therefore not necessary. Complete conversion of oxalate was checked in all samples by adding a tracer amount of $[^{14}C]$oxalate and measuring the radioactivity left in the medium.

Standard Curve

The effect of standard solutions of $NaHCO_3$ and oxalate are shown in Fig. 2. The conductivity measured for oxalate or $NaHCO_3$ at equal molar concentrations were, as expected, not found to be different.

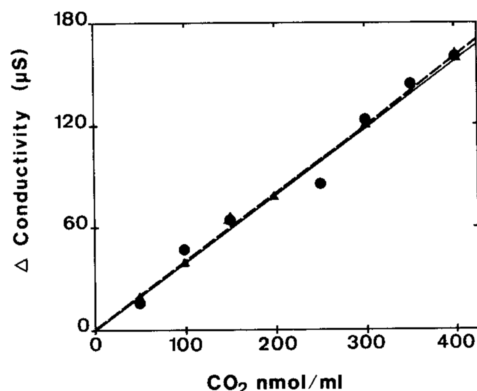

Fig. 2. *Standard Curve.* Different amounts of sodium bicarbonate or oxalate were incubated. The CO_2 formed was absorbed in $Sr(OH)_2$ and the change in conducitivity measured. ●—● Oxalate, ▲—▲ $NaHCO_3$

The standard curves were linear up to an oxalate concentration of 400 nmol ml^{-1}. The mean decrease in the conductivity for 50 nmol ml^{-1} increase of the oxalate concentration was 20.6 μS ± 0.4 μS (mean ± S.E.M. of 19 standard curves).

The limit of detection (Bμ) was calculated from the formula $B\mu = 2 \times 1.645 \sqrt{S_B^2 + S_A^2}$ [17], where S_B is the standard deviation of the blank values and S_A the standard deviation of the lowest oxalate concentration tested (50 nmol ml^{-1}). Bμ was found to be 23 nmol ml^{-1} oxalate for the standard curve.

Determination in Urine

Recovery. 100 nmol ml^{-1} of oxalate were added to 32 urine specimens of morning urines and the recovery was found to be 93.3 ± 2.5% (mean ± S.E.M.). The radioactivity measured after incubation with enzyme was 49.7% ± 0.4 (mean ± S.E.M.) (range 46.5%–54.2%) of the amount added.

Specificity. Glyoxalate, pyruvate, succinate, urea and uric acid at supraphysiological concentrations were added to urine samples and the urine analysed for oxalate. As seen from Table 2, none of the substances had an effect on the assay.

Precision. Five urine samples from different persons were analysed for oxalate repeatedly on consecutive days. Four to seven analyses were performed per urine with a total of 28 determinations. A mean oxalate concentration was calculated for each urine sample (range from 123.2 to 239.9 μmol l^{-1}) and taken als 100%. Each single value was expressed as percentage of its corresponding mean. The percentage values were pooled and the reproducibility, expressed as the standard deviation of the individual values, was found to be 12.6% (n = 28) (range: 81.2%–122.4%).

Limit of Detection Bμ. Bμ was calculated as for the standard curve [17]. S_B is the standard deviation of the blank values in urine and S_A the standard deviation of values of urine with a low oxalate concentration. Such urines were obtained by precipitating calcium oxalate with calcium sulphate and ethanol [23]. Bμ was found to be 35 nmol ml^{-1} oxalate for urine.

Sensitivity. 1 ml of urine was usually sufficient to give adequate sensitivity. This volume could be doubled when the oxalate concentration in the urine was low. This low amount of urine permitted the use of small amounts of enzyme.

Normal range. The mean 24 h urine oxalate excretion of 11 healthy male and 16 female laboratory staff on unrestricted diets was 240 ± 20 μmol (mean ± S.E.M.). The range was 150 to 500 μmol. In 8 h 15–18 samples can be analysed with this method.

Table 3 shows the 24 h urinary oxalate excretion obtained by this method in comparison with the 24 h oxalate excretion obtained by others with generally different methods. The mean 24 h oxalate excretion that was found with our method is slightly lower than has been reported for most other methods. The range, which is comparable with the other methods, indicates that this is not due to an insensitivity of the method.

Table 2. Specificity of oxalate decarboxylase

Test substance	Concentration	Number of urines	Oxalate % of control	S.D.	
Glyoxalate	70 μM	9	99.2	3.2	N.S.
Pyruvate	100 μM	9	101.0	6.7	N.S.
Succinate	75 μM	7	103.7	4.9	N.S.
Urea	250 mM	7	94.9	4.8	N.S.
Uric acid	9 mM	3	102.7	2.0	N.S.

Urine samples were analysed for oxalate with or without addition of test substance. The oxalate concentration found with test substances is expressed as percentage of the value found without the test substance (control). Student's t-test with a hypothetical mean (μ) of 100% for the urine samples without added substance was applied

$$t = \frac{[\bar{x} - \mu]}{\text{S.D.}} \sqrt{n}.$$

Table 3. 24 h urinary oxalate excretion obtained by different methods

Isolation of oxalate from urine	Kind of determination	n Subjects	Range mmol/24h	Mean mmol/24h	Reference
Extraction	Fluoremetric	60	0.10–0.32	–	[23]
Precipitation	Colorimetric	22	0.19–0.48	0.34	[1]
Precipitation	GC[c]	150	0.08–0.50	0.28	[3]
Precipitation	GC	8	0.17–0.58	0.40[a,b]	[4]
Precipitation	GC	8	0.10–0.26	0.18	[5]
Precipitation	GC	40	0.10–0.49	0.27	[6]
None	Enzymatic; oxalate decarboxylase	22	0.14–0.46	0.30	[9]
None	Enzymatic; oxalate decarboxylase	22	0.16–0.42	0.29	[10]
None	Enzymatic; oxalate oxydase	6	0.05–0.42	0.26[a]	[11]
None	Enzymatic; oxalate decarboxylase	37	0.15–0.50	0.24	This paper

[a] assumed anhydrous for calculation in mmol [b] mean over 5–7 days [c] GC; gas chromatography

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