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Determination of Oxalate in Urine Using Oxalate Oxidase: Comparison with Oxalate Decarboxylase

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Dedicated to Prof. Dr. Dr. Ernst Schütte on the occasion of his 70th birthday

Summary: The oxalate content of urine is determined by means of oxalate oxidase and simple pH measurement. The enzyme specifically decarboxylates oxalate, producing two moles CO_2 per mole oxalate. The CO_2 diffuses into an alkaline buffer solution (Hallson, P. C. & Rose, G. A. (1974), Clin. Chim. Acta *55*, 29–39) in the closed reaction vessel, and reduces the pH value, which is measured with an electrode. Only 125 µl native urine is required to measure oxalate concentrations in the range of 80 µmol/l to 1.6 mmol/l (corresponding to 7 to 144 mg anhydrous oxalic acid per liter). The limit of detection is 10 nmol oxalate, and the accuracy is 101% with a coefficient of variation of 6%.

The method described is insensitive to various interfering factors, such as reducing and oxidizing substances, cloudy or colored samples. It is therefore also suitable for oxalate determination in food technology and plant breeding.

Bestimmung von Oxalat in Urin mit Oxalat-Oxidase: Vergleich mit Oxalat-Decarboxylase

Zusammenfassung: Der Oxalatgehalt von Urin wird mittels Oxalat-Oxidase durch einfache pH-Messung bestimmt. Das Enzym decarboxyliert spezifisch Oxalat, wobei je Mol Oxalat zwei Mol CO_2 entstehen. Durch Diffusion des CO_2 in eine alkalische Pufferlösung (Hallson, P. C. & Rose, G. A. (1974), Clin. Chim. Acta *55*, 29–39) in geschlossenen Reaktionsgefäßen erniedrigt sich der pH-Wert, der mit einer Elektrode gemessen wird. Dabei genügen 125 µl Nativ-Urin, um Oxalatkonzentrationen im Meßbereich von 80 µmol/l bis 1,6 mmol/l (entsprechend 7 mg bis 144 mg wasserfreie Oxalsäure je l) zu erfassen. Die Nachweisgrenze liegt bei 10 nmol Oxalat, die Richtigkeit beträgt 101% bei einem Variationskoeffizienten von 6%.

Die beschriebene Methode zeichnet sich durch geringe Störanfälligkeit gegenüber verschiedenartigen Analysenproben aus. Reduzierende und oxidierende Substanzen, trübes und gefärbtes Untersuchungsmaterial stören nicht. Sie ist deshalb auch für Oxalatbestimmungen in der Lebensmitteltechnologie und Pflanzenzüchtung geeignet.

Introduction

The determination of oxalate in urine has become clinically important for the recognition and treatment of various forms of hyperoxaluria. In addition, the oxalate excretion of patients with calcium oxalate-lithiasis has come into the center of attention in clinical urinary stone research. Outside of medicine, an exact and simple method for oxalate determination is needed in food technology and plant breeding.

Many methods have been described in the literature; *Hodgkinson* (2) provides a review. All previously described enzymatic techniques are based on the enzyme oxalate decarboxylase (oxalate carboxy-lyase, EC 4.1.1.2), which converts oxalate to CO_2 and formate:

$$(COOH)_2 \xrightarrow{\text{decarboxylase}} CO_2 + HCOOH$$

Each of the reaction products can be quantitatively measured by well-known methods (3).

If CO_2 is to be measured, the use of oxalate oxidase (oxalate: oxygen oxidoreductase, EC 1.2.3.4), which has not previously been used for oxalate determination

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seems advantageous, because it produces two moles CO_2 from one mole oxalate (4):

$$(COOH)_2 + O_2 \xrightarrow{\text{oxidase}} 2CO_2 + H_2O_2$$

Furthermore, the enzyme catalase (hydrogen-peroxide: hydrogen-peroxide oxidoreductase, EC 1.11.1.6) can be used to decompose the H_2O_2 produced:

$$H_2O_2 \xrightarrow{\text{catalase}} 1/2O_2 + H_2O$$

The coupled reaction thus achieves a rapid, quantitative conversion of oxalate, and requires only half as much oxygen as the oxidase reaction by itself:

$$(COOH)_2 + 1/2O_2 \xrightarrow{\text{oxidase, catalase}} 2CO_2 + H_2O$$

We used this reaction as a basis for the development of a method for oxalate determination in urine. The reaction takes place in a closed vessel in a buffer with a pH value of 3.8. The CO_2 is released from the acidic buffer and diffuses into an alkaline buffer solution and causes its pH value to decrease (1), in linear proportion to the amount of oxalate present.

Materials and Methods

Reagents

All chemicals were obtained in p. a. grade from Merck AG (Darmstadt). Bovine liver catalase, about 65 000 U/mg, corresponding to 1.3 GU/l (Boehringer Mannheim GmbH), was diluted before use 1: 5 with succinate buffer (see below). We used oxalate decarboxylase from Worthington, and we purified oxalate oxidase from barley seedlings (5). The purification steps were extraction with H_2O, heat denaturation of undesired proteins, fractional ammonium sulfate precipitation (45–65%) and ion-exchange chromatography on DEAE-Sephadex A-50, with imidazole buffer, I = 0.05, pH 8. The enzyme was eluted with a NaCl gradient. From 1 kg seedlings we obtained 6 mg protease- and catalase-free enzyme protein with a specific activity of about 15 U/mg (37 °C, succinate buffer, pH 3.8). Partially purified enzyme preparations containing catalase activity also suffice for oxalate determination, but for reasons of stability, they should not contain protease. The preparation was stable as a solution in succinate buffer (2 g/l) containing merthiolate (20 mg/l) for at least 3 months at 4 °C. Before use it was diluted with succinate buffer (see below) 1: 40. Therefore the applied 400 µl contained 20 µg enzyme protein (about 0.3 U). Since many factors affect the determination of enzyme activity, the amounts of enzyme used are given here as µg protein of our preparation.

Solutions

All solutions were prepared with doubly distilled water from which CO_2 had been removed by vacuum distillation. O_2 was bubbled through for 30 min (1 l/min) before use.

I. 50 mmol/l succinate buffer, pH 3.8 + 5 mmol/l EDTA: Dissolve 5.9 g succinic acid + 1.86 g EDTA in about 900 ml H_2O, adjust to pH 3.8 with 1 mol/l NaOH and fill to 1 l with H_2O.

II. Alkaline buffer pH 10:
Stock solution: 4.8 g Na_2CO_3 + 6.7 g $NaHCO_3$ dissolved in 100 ml H_2O. Before use, dilute 1: 800 with H_2O.

III. Oxalate standard solution (100 µmol/l):
Dissolve 12.6 mg oxalic acid · $2H_2O$ (M_r 126.07) in about 900 ml succinate buffer. Adjust to pH 3.8 with 1 mol/l NaOH, then fill to 1 l with buffer.

Equipment

pH meter, readable to 0.01 pH.

Micro combined pH electrode

Optional: shaking apparatus for the reaction vessels. We used the thermostatic shaking bath from B. Braun (Melsungen), but without the thermostat.

Oxygen pressure bottle with a reducing valve.

Conical *Warburg* reaction flask from B. Braun (Melsungen), No. 830 415, with a total volume of about 13 ml. The central inner well holds a shortened Eppendorf-Netheler cup (Eppendorf Gerätebau, Hamburg, No. 3810) for the alkaline buffer solution. This plastic cup has a small piece of tubing pulled over its conical end, so that it sits more firmly in the central well. The main glass joint is closed with a stopcock, bore 1.5 mm (fig. 1). Simpler flasks without side arms are less suitable, because then oxalate oxidase is already present in the assay solution during the gassing with O_2. This means that CO_2 produced during gassing escapes measurement.

Procedure (tab. 1)

For oxalate determination, 125 µl fresh, filtered urine from a sample collected over a known period is placed without pretreatment in the main chamber of the *Warburg* reaction flask, along with 2 ml succinate buffer (I) and 5 µl = 1.3 kU catalase. The central inner well contains 400 µl alkaline buffer (II), and the side arm holds 400 µl oxalate oxidase solution (20 µg protein). The flask is gassed through the open stopcock and capillary stopper with 0.25 l O_2/min, then the stopcocks, which are sealed with silicone grease, are closed. The flask is briefly inclined so that the enzyme flows out of the side arm into the main chamber. The oxalate is then converted to CO_2, which diffuses into the alkaline buffer during the incubation at room temperature for at least 16 h, and reduces its pH value. At the end of the incubation time, the central inner vessel can be removed and the pH value measured.

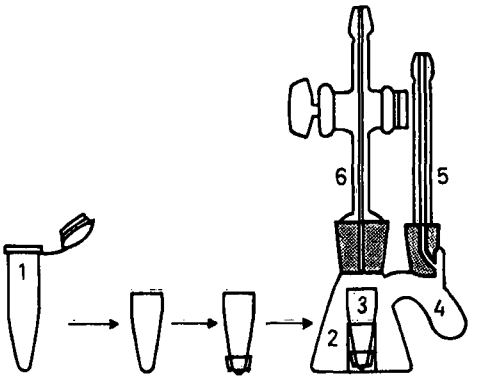

Fig. 1. Conical *Warburg* flask used for oxidative decarboxylation of oxalate.
1: Eppendorf-Netheler Cup, 2: main chamber (urine, buffer, catalase), 3: central inner well (alkaline buffer), 4: side arm (oxalate oxidase), 5: capillary stopper, 6: stopcock, bore 1.5 mm.
All vessels are gassed for 10 min with O_2 (0.25 l/min). Then the stopcocks are closed, and the solution from the side arm is tipped into the main chamber. The flask is gently shaken at room temperature. After at least 16 h incubation, the pH of the alkaline buffer in the central inner well is measured.
If a pH difference of 0.64 is obtained for 100 nmol oxalate, the oxalate concentration is

$$(\overline{pH}_{blank} - \overline{pH}_{assay}) \times \frac{100}{0.64} \times 8 \left[\frac{\mu mol}{l}\right]$$

Tab. 1. Scheme of oxalate determination in *Warburg* flasks. Each experiment is carried out in duplicate.

		Blank	Assay	Control
	All solutions gassed with O_2 (1 l/min) for 30 min			
Main chamber	Succinate buffer (I) [µl]	2000	2000	1000
	Standard oxalate solution (III) [µl]	–	–	1000
	Urine [µl]	125	125	125
	Catalase [µl]	5	5	5
Side arm	Succinate buffer (I) [µl]	400	–	–
	Oxalate oxidase [µl]	–	400	400
Central inner well	Alkaline buffer (II) [µl]	400	400	400

In addition to this pH value, an individual sample blank value must be determined. This pH value varies with the O_2 gassing in different runs. The difference between sample and blank pH is then determined, and the oxalate content of the sample is calculated from a calibration curve.

Results

Figure 2 shows the linearity of the relationship between pH difference and oxalate content up to 200 nmol per sample: y = 10.142 - 0.0064 x. With the samples of 125 µl urine used in this procedure, oxalate concentrations of 80 µmol/l to 1.6 mmol/l (7.2 mg/l to 144 mg/l anhydrous oxalic acid, M_r 90.04) are directly measurable.

In our experience, it is sufficient to set up a calibration curve once, since the same reproducible pH difference of 0.64 per 100 nmol oxalate is obtained. Furthermore, this can be checked by a standard control value for each sample. This is obtained by adding exactly 100 nmol oxalate to the urine sample. However, it is not necessary to determine such a standard control value for every single urine sample; it is enough to make one check for each experimental series to test the system.

Blank values for enzymes and for oxalate were negligible with our preparation; the latter did not change with various amounts of exogenously added oxalate.

Effect of catalase

As explained in the introduction, the catalase added to the mixture decomposes the H_2O_2 generated in the oxalate oxidase reaction. This accelerates the reaction and halves the amount of oxygen required. It can be seen from the experiment shown in figure 3 that the addition of catalase can reduce the amount of oxalate oxidase required. We therefore added 1.3 kU catalase to each sample. In principle, however, catalase is not needed if sufficient oxalate oxidase activity is present.

Amount of oxalate oxidase

Urine contains numerous cations and anions which reduce the activity of the oxalate oxidase (6). Therefore more enzyme must be added to biological samples than to aqueous solutions. As can be seen from figure 4, a quantitative determination of urine oxalate is only possible under the given experimental conditions when at least 20 µg oxalate oxidase is added. This amount has been sufficient in every urine sample so far examined, as was determined from the quantitative recovery of 100 nmol added oxalate.

Time required for diffusion

To determine the amount of time necessary for diffusion, the oxalate in the main chamber was replaced by 150 nmol Na_2CO_3, so that all the CO_2 would be imme-

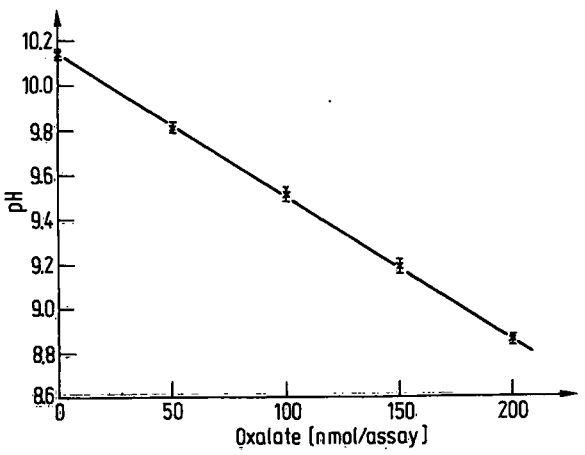

Fig. 2. Calibration curve for oxalate determination, $\bar{x} \pm SD$, n = 3. CO_2 is generated from oxalate by oxalate oxidase and proportionately reduces the pH value of an alkaline buffer. 100 nmol oxalate correspond to a pH difference of 0.64.
Regression plot: y = 10.142 – 0.0064 x, r = 0.9991
S_{yx} = 0.02068

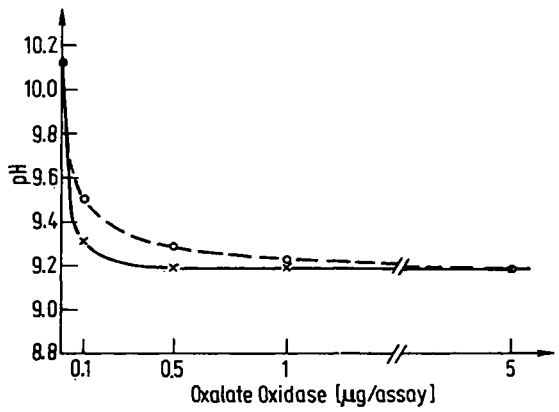

Fig. 3. Reduction of the amount of oxalate oxidase needed by addition of catalase. pH value after 16 h incubation of 150 nmol oxalate as a function of oxalate oxidase and with added catalase.
x = with catalase, 1.3 kU/assay
o = without catalase

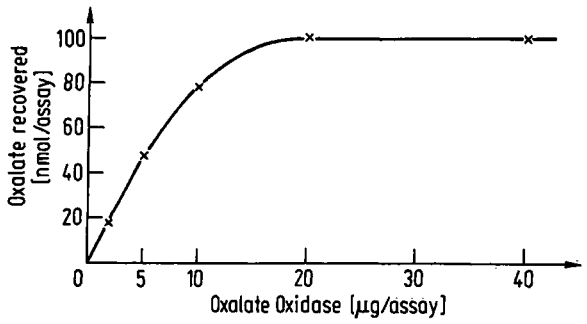

Fig. 4. Recovery of oxalate.
100 nmol oxalate was added to a pooled urine sample and the recovery was plotted as a function of oxalate oxidase.

Tab. 2. Oxalate contents in urine of normal persons.
Each value is the mean of duplicate determinations.

Urine sample	Oxalate concentration [µmol/l]	Oxalate excretion [µmol/24 h]	Recovery of 100 nmol oxalate [nmol]
1	345	418	109
2	258	357	100
3	123	53	102
4	266	141	98
5	197	395	92
6	123	223	103
$\bar{x}$ ± SD	219 ± 88	265 ± 149	100.7 ± 5.7 CV = 5.6%

diately released on addition of H_2SO_4 instead of enzyme from the side arm. The other conditions were the same as in the regular determination. The experiment served to measure the time required for diffusion independently of the enzymatic formation of CO_2. Figure 5 shows that the pH value of the alkaline buffer becomes constant only after 16 h. The oxalate cannot be more rapidly analysed with this system, and the time required for diffusion is prolonged by about 1/3 when the incubation flasks are not shaken.

The length of time required for diffusion is advantageous, however, for the pH measurement after incubation, because the CO_2 from the air has no effect during the time required for this procedure.

Urine oxalate values in normal subjects

The oxalate concentration in the urine of 6 healthy persons was measured (tab. 2). The values obtained ranged from 123 to 345 µmol/l.

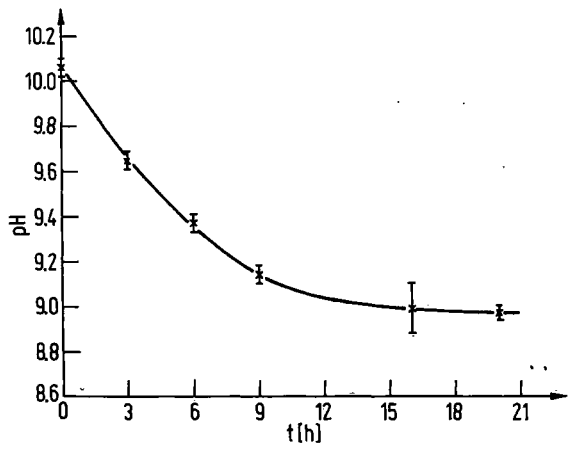

Fig. 5. Dependence of the measured pH value on the diffusion time. At the beginning of the experiment, CO_2 was released from 150 nmol Na_2CO_3 with H_2SO_4.
$\bar{x}$ ± SD (n = 3).

Criteria of the method

We determined the following values as criteria of the method:

1. *Limit of detection*

The limit of detection according to *Kaiser* (7) is 10 nmol oxalate.

2. *Specificity*

The enzyme oxalate oxidase converts only oxalate, specifically. No other substrates could be found.

3. *Accuracy*

100 nmol oxalate was added to each of 6 different samples of urine. On the average 100.7 ± 5.7 nmol was recovered (lowest value 92, highest value 109). Coefficient of variation = 5.6% (see table 2).

4. *Reproducibility*

The reproducibility among a series of n = 6 was 266 ± 14 µmol/l, corresponding to a coefficient of variation of 5.3%. From day to day, with n = 5, we found 257 ± 19 µmol/l, corresponding to a coefficient of variation of 7.4%.

Discussion

Oxidation of oxalate by oxalate oxidase produces 2 mol CO_2, while oxalate decarboxylase yields only 1 mol CO_2. If CO_2 is measured as the reaction product of an enzymatic oxalate conversion, then the sensitivity with oxalate oxidase should be twice that with oxalate decarboxylase, i. e. the pH difference measured with the present technique should be twice as great. Using our experimental set up we measured the pH changes in the alkaline buffer as a function of the amount of oxalate (fig. 6) with each of the two enzymes. As expected, the pH difference with oxalate oxidase is twice that with

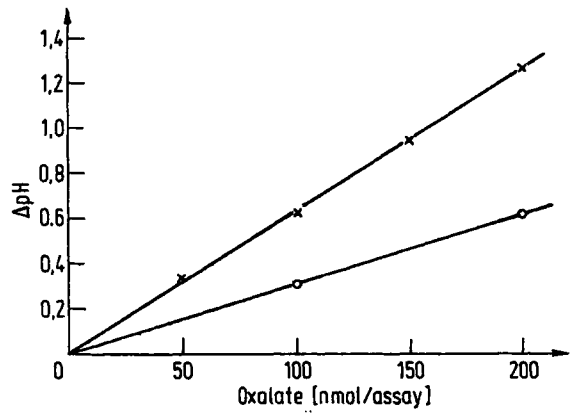

Fig. 6. Comparison of oxalate determination with oxalate oxidase and oxalate decarboxylase. 20 μg oxalate oxidase or 0.37 U oxalate decarboxylase was added to convert the oxalate. The pH difference measured with oxalate oxidase is twice that with oxalate decarboxylase, since the former produces twice as much CO_2.
x = Oxalate oxidase
o = Oxalate decarboxylase

oxalate decarboxylase and is 0.64 for 100 nmol oxalate, compared to 0.32. The limit of detection was reduced in this way to 10 nmol oxalate. Since the reaction flask holds a maximum of 3 ml sample volume, this corresponds to a minimal oxalate concentration in the sample to be analysed of 3 μmol/l.

This limiting concentration is so low that it appears possible to analyse serum, which has an oxalate concentration of 15–30 μmol/l. However, prior to analysis 25 mmol endogenous CO_2/l would have to be removed.

The low limit of detection and the wide range of 10–200 nmol oxalate make it possible to dilute urine by maximally 1:25. We used a dilution of 1:20. The ions which inhibit oxalate oxidase activity (6) are thus sufficiently dilute that economical amounts of enzyme can be used. In addition, the concentrations, and thus effectiveness of other possible interfering substances, such as previously unknown inhibitors of oxalate oxidase, are reduced. In principle, the method described, measurement of CO_2 released from oxidation of oxalate after diffusion into an alkaline buffer, is not very sensitive to interference. Neither oxidizing nor reducing substances interfere, and cloudy or colored samples can be used. This makes the technique independent of the type of sample. It can be used not only for urine, but also for determination of oxalate in food technology and plant breeding.

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