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Determination of plasma oxalate with oxalate oxidase

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Summary

A method for the determination of plasma oxalate using oxalate oxidase (EC 1.2.3.4) and deproteinised plasma is described. Reference values are 1.2–6.4 μmol/l ($n = 24$, mean ± SD 3.3 ± 1.5 μmol/l). The sensitivity is 6–7 nmol, the accuracy 3–5 nmol, and the coefficient of variation 10.4% (at a level of 23 nmol). The recovery from plasma spiked with oxalate was 105 ± 8% ($n = 8$). Allowing blood to stand for 4 h at room temperature had no effect on plasma oxalate levels; inhibitors of glyoxylate converting enzymes interfered with oxalate oxidase.

Introduction

A large number of techniques for the determination of oxalate in plasma or serum have been published (Table I). For most of the in vitro methods [1–13], the reported normal reference values are rather high compared with those obtained with in vivo [^{14}C]oxalate clearance methods [14–18]. Recently, however, two in vitro methods with a lower range of normal values were published [12,13]. The present report describes a third technique, making use of the enzyme oxalate oxidase, which has the advantage of liberating two mol CO_2 per mol oxalate. Because of the controversy about the in vitro generation of oxalate by glyoxylate converting enzymes and the need to use inhibitors of these enzymes [12,13], we investigated the influence of addition of inhibitors, and of delayed processing of the blood.

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TABLE I

Reported normal reference values for plasma oxalate (in μmol/l)

Authors	Year	Ref.	Range	Mean	SD	*n*	Technique
Chambers and Russell	1973	1	-	161	99	20	GC
Yao et al	1975	2	33–111	-	-	-	Enz/Pot
Crawhall and Watts	1961	3	< 45	-	-	22	Enz/Gas
Hatch et al	1977	4	8–52	20.3	10.1	40	Enz/Spect
Sugiura et al	1980	5	7–46	-	-	50	Enz/Col
Gelot et al	1980	6	9–41	20.0	8.8	40	GC
Zarembski and Hodgkinson	1965	7	15–31	-	-	15	Fluor
Krugers et al	1976	9	13–28	-	-	20	Col
Nuret and Offner	1978	9	-	16.0	4.5	20	GC
Knowles and Hodgkinson	1972	10	9–16	13.1	2.7	20	Enz/Col
Bennett et al	1979	11	5–17	9.8	3.1	15	Rad
Present study	1983	-	1.2–6.4	3.3	1.5	24	Enz/Pot
Wolthers and Hayer	1982	12	1.3–5.3	2.8	1.1	22	GC
Akcay and Rose	1980	13	0–5.4	2.3	1.7	20	Enz/Pot
Williams et al	1971	14	1.4–2.3	2.0	0.6	6	Isot
Pinto et al	1974	15	0.4–2.8	1.4	0.3	6	Isot
Hodgkinson and Wilkinson	1974	16	1.3–1.6	1.4	0.2	3	Isot
Prenen et al	1982	17	1.0–1.8	1.4	0.3	10	Isot
Constable et al	1979	18	0.8–2.1	1.3	0.5	8	Isot

Abbreviations: GC, gas chromatographic; Enz, enzymatic; Pot, potentiometric; Gas, gasometric; Fluor, fluorimetric; Spect, spectrophotometric; Col, colorimetric; Rad, radioenzymatic; Isot, in vivo isotope clearance.

Materials and methods

Reagents

All chemicals were purchased in p.a. grade from Merck AG (Darmstadt, FRG). Bovine liver catalase (EC 1.11.1.6, 65 000 U/mg) was obtained from Serva GmbH (Heidelberg, FRG), diluted 1 : 9 (130 000 U/ml), and stored in portions at −20°C. Oxalate oxidase (EC 1.2.3.4) was purified from barley seedlings by the method of Kohlbecker et al [19] and Chiriboga [20]. The final purification was done on a 2.6 × 40 cm DEAE-Sepharose CL-6B column (Pharmacia, Uppsala, Sweden), and enzyme activity in the fractions was assessed with guajacol and peroxidase (EC 1.11.1.7) [21]. The final solution contained 6.4 U/ml oxalate oxidase, specific activity 22.2 U/mg, and was stored in portions at −20°C.

The following solutions, which were prepared from CO_2-free, O_2-saturated deionised water, were used for the determinations; succinate buffer (freshly prepared): succinic acid 50 mmol/l, EDTA di-sodium salt 5 mmol/l, merthiolate 0.025 mmol/l, pH adjusted to 3.8 with 1 mol/l NaOH; alkaline buffer (stock solution): sodium carbonate 216 mmol/l, sodium hydrogen carbonate 384 mmol/l, diluted 1 : 1000 before use (final concentration 0.60 mmol/l, pH 9.8); and oxalate standard solution: oxalic acid dihydrate 1 mmol/l.

For the enzyme inhibitor experiments, the following solutions were used: boric

acid 700 mmol/l, TRIS 420 mmol/l, DL-β-phenyllactic acid 280 mmol/l, sodium pyruvate 280 mmol/l, potassium iodate 42 mmol/l, and allopurinol 17.5 mmol/l. 1 vol. of these solutions was combined with 6 vols. of succinate buffer before use.

Equipment

For the incubations, use was made of conical 50-ml flasks provided with a B 19/26 socket and a solid stopper, and with a 35 × 10 mm inner cylinder mounted at the bottom. A Radiometer type PHM 62 (Copenhagen, Denmark) equipped with an Ingold type HA-405-M8 (Zürich, Switzerland) combined glass electrode was used for pH measurements. All manipulations were carried out in an Isolette model C-77 incubator (Hatboro, PA, USA).

Procedures

Preparation of blood samples. Fifty milliliters of heparinised blood was centrifuged for 10 min at 2000 × *g* and 4°C immediately after collection. Plasma was deproteinised [13] by adding an equal volume of 0.05 mmol/l HCl and heating in a waterbath at 100°C for 5 min. After centrifugation for 30 min at 20000 × *g* and 4°C, the supernatant was adjusted to pH 3.8 with 1 mmol/l HCl, merthiolate was added to a concentration of 0.025 mmol/l, and the material was stored at −20°C. After storage for at least 6 months, there was no increase or decrease in the measured oxalate content. Addition of [^{14}C]oxalate prior to deproteinisation resulted in a recovery of 98 ± 2% ($n = 6$) of the radioactive label in the supernatant. Because oxalate is not metabolised by plasma [22], this finding may be interpreted as a complete recovery of oxalate.

Determination of oxalate. Measurements in deproteinised plasma and corresponding blanks were performed in duplicate. The outer compartments of the flasks were filled with 10–15 ml deproteinised plasma, the volume was made up to 20 ml with succinate buffer, and 1300 U catalase were added. Measurements of reagent blanks and standards were performed in triplicate: the outer compartments were filled with 20 ml succinate buffer with or without addition of standard solution (100 nmol), followed by addition of catalase. The inner cylinders were provided with 1 ml alkaline buffer and the reaction was started by addition of 0.25 U of oxalate oxidase to all flasks except the plasma blanks. After incubation for 72 h at 20 ± 1°C, the pH of the alkaline buffer was measured. Shorter incubation times led to smaller pH decreases. The plasma oxalate concentrations (nmol/ml or μmol/l) were calculated using the formula:

$$P_{Ox} = \frac{(\text{pH plasma blank} - \text{pH deproteinised plasma})}{(\text{ml deprot. plasma}/2) \times (\text{pH reagent blank} - \text{pH standard})/100}$$

Results

Linearity

As Fig. 1 shows, the relationship between pH and amounts of oxalate added to succinate buffer appeared to be linear up to at least 100 nmol.

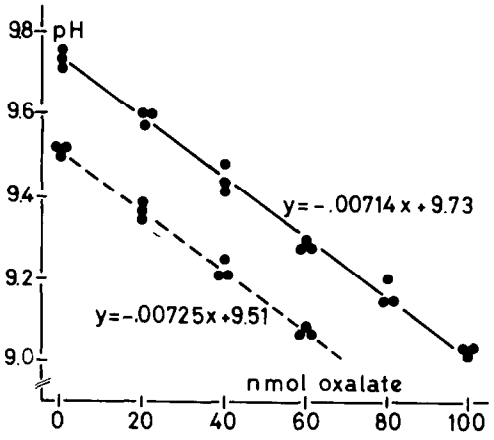

Fig. 1. Decrease in pH of alkaline buffer after addition of standard oxalate to succinate buffer (solid line) and deproteinised plasma (dashed line).

Parallellism

Fig. 1 also shows that the addition of oxalate to deproteinised plasma gave rise to a standard line with the same slope as that of the external standard line.

Accuracy

The values obtained in a large number of determinations were used to calculate the mean of the standard deviations for triplicate standards and reagent blanks ($n = 47$) and duplicate unknowns and plasma blanks ($n = 98$) (Table II). Comparison of the variances by Bartlett's homogeneity test points to heteroscedasticity ($\chi^2 = 15.2$, df = 3, $p < 0.002$). Calculation of the variance ratio, F, for reagent blanks and standards showed no significant difference; the same holds for the variance ratio of unknowns and plasma blanks. The F ratio of the combined variance of reagent blanks plus standards and combined variance of unknowns plus plasma blanks reaches significance (Table II).

TABLE II

Variance ratios for standard deviations in reagent blanks, standards, unknowns and plasma blanks

Samples	Mean SD	F	df	p
Reagent blanks	0.026			
Standards	0.024	1.17	46, 46	> 0.10
Combined	0.025			
Unknowns	0.019			
Plasma blanks	0.017	1.25	97, 97	> 0.10
Combined	0.018	1.93	91, 193	< 0.001

Abbreviations: SD, standard deviation (in pH-units); F = variance ratio; df, degrees of freedom; p, significance.

TABLE III

Recovery of standard oxalate after addition of 6.7 μmol/l to 5 plasma samples

Plasma	Standard oxalate recovered (μmol/l)		
	control	expected	measured
A	2.1	8.8	8.6
B	2.3	9.0	9.4
C	3.4	10.1	11.1
D	3.7	10.4	11.4
E	3.8	10.5	9.9
Mean	3.1	9.8	10.1

Sensitivity

If a pH decrement of 0.7–0.8 pH units is assumed for the standard of 100 nmol (see also Fig. 1) and $2 \times SD = 0.05$ for the reagent blank, calculation yields a sensitivity of 6–7 nmol.

Precision

Table III shows the results of addition of a known amount of oxalate to different plasmas, and Fig. 2 of addition of increasing amounts of oxalate to a single plasma. Overall, the mean recovery amounted to 105 ± 8% ($n = 8$).

Reproducibility

One plasma with a mean concentration of 7.7 μmol/l was analysed seven times over a 4-month period; for these determinations, 6 ml of deproteinised plasma was used instead of 10–15 ml, which corresponds to an actual measured amount of 23.1 ± 2.4 nmol oxalate. At this level, the coefficient of variation was 10.4%.

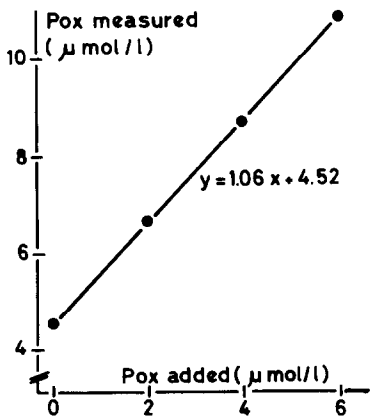

Fig. 2. Recovery of standard after addition to plasma in increasing amounts.

TABLE IV

Effect of delayed processing of blood on plasma oxalate (in μmol/l)

Plasma	Time		
	0 h	2 h	4 h
F	3.7	4.0	3.7
G	5.3	5.0	4.7
H	5.5	5.5	5.0
I	12.3	10.6	12.0
Mean	6.6	6.6	6.4

Time effects

The effect of delayed processing of samples was studied by dividing each of four samples of blood into three portions. One portion was used immediately, and the other two after standing at room temperature for 2 and 4 h. The results (Table IV) show that plasma oxalate does not tend to decrease or increase significantly with time.

Enzyme inhibitors

The effect of inhibitors of xanthine oxidase (EC 1.2.3.2), lactate dehydrogenase (EC 1.1.1.27), and glycollate oxidase (EC 1.1.3.1) was assessed by adding these inhibitors to succinate buffer and measuring the changes in pH of the alkaline buffer in the presence and absence of oxalate oxidase in the incubation mixture. As Table V shows, some of the inhibitors interfered with the determination: two of them served partially as a substrate for oxalate oxidase, which led to CO_2 generation, and two others gave non-enzymatic pH decreases. This was also the case for a combination of the three least interfering inhibitors. On the basis of these findings, no attempt was made to evaluate the effect of the addition of inhibitors to whole blood.

TABLE V

Effect of enzyme inhibitors on pH of alkaline buffer

Enzyme to be inhibited	Inhibitor	pH decrease with oxalate oxidase		Effect *
		absent	present	
Xanthine oxidase	Allopurinol	0.00	0.16	±
Lactate dehydrogenase	Borate	0.67	0.60	+
	Iodate	0.00	0.00	−
	Pyruvate	0.70	0.74	+
Glycollate oxidase	Phenyllactate	0.06	0.54	+
	TRIS	0.00	0.00	−
All 3 enzymes	All + Iod + TRIS	0.95	1.08	+

* + and −, interfering effect present or absent, respectively.

Reference values

In a group of 24 apparently healthy fasting subjects (14 males, 10 females), the mean concentration of plasma oxalate was 3.3 ± 1.5 μmol/l with a range of 1.2–6.4 μmol/l.

Discussion

Plasma oxalate levels determined by the technique described here give normal values that are among the lowest reported in the literature for chemical methods. The method provides good linearity and parallelism, and the precision and reproducibility are adequate.

Although the accuracy was slightly lower for standards (mean SD of 0.025 pH units) than for samples (mean SD of 0.018 pH units), the pH decrease is generally smaller for samples than for standards, which makes the relative SD larger for samples than for standards. This in turn makes the SD for the actual measured amount of oxalate (see also the equation in 'Methods') almost independent of that amount, especially in the lower concentration range. For example, if a typical pH decrease of 0.75 is assumed for the standard (see Fig. 1) and 0.15 for a sample containing 20 nmol oxalate (e.g. 10 ml deproteinised plasma with a concentration of 4 μmol/l plasma), an SD of ±3.5 nmol is obtained (denominator ±17%, nominator ±4.7%), i.e. ±0.7 μmol/l. For samples containing 5, 10, 40 and 80 nmol, the calculated values for the SD are 3.4, 3.4, 3.9 and 5.1 nmol, respectively.

The sensitivity depends mainly on the volume and strength of the alkaline buffer. In our hands, the use of 1.0 ml buffer with a concentration of 0.06 mmol/l carbonate/bicarbonate gave optimal results. At a detection limit of 6–7 nmol, the use of 15 ml deproteinised plasma permits the determination of plasma concentrations of 0.8–0.9 nmol/l, which is below our range of normal reference values.

Akcay et al [13] have advocated the use of inhibitors for enzymes capable of oxidising glyoxylate, because blood can generate oxalate spontaneously on standing. As inhibitors, these authors used boric acid, DL-β-phenyllactic acid, and allopurinol, none of which interfered with their enzyme, i.e., oxalate decarboxylase (EC 4.1.1.2). However, these and other inhibitors gave substantial interference when the enzyme oxalate oxidase was used (Table V). Our experiment with delayed processing of blood samples shows that the need for the use of inhibitors is questionable, because plasma oxalate levels remained constant up to 4 h of standing at room temperature (Table IV). These findings are in agreement with those of Wolthers and Hayer [12], who studied the effect of standing time on serum.

Finally, some remarks must be made in this context on the use of electrodes. We have tested several makes of combined glass electrodes and found most of them to be unstable or to react too slowly in the higher pH range. Only the Ingold HA-405-M8 and the Russell TR-CMAWL-6-TB were suitable for our purposes. With this restriction, the method presented here is a convenient one, requiring only simple equipment.

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