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The Fluorimetric Determination of Oxalic Acid in Blood and Other Biological Materials

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1. Oxalic acid is separated from interfering substances by extraction with tri-*n*-butyl phosphate followed by co-precipitation with calcium sulphate. The precipitated oxalic acid is then reduced to glyoxylic acid, which is coupled with resorcinol to form a coloured fluorescent complex. 2. The spectrofluorometric method described is sensitive and highly specific, the minimum detectable amount of oxalic acid being 0·9 μmole under the recommended conditions. 3. The concentration of oxalic acid in blood from 15 normal adults was 200–320 μg./100ml. For serum the range was 135–280 μg./100ml. The urinary excretion of oxalic acid by 60 normal adults on a normal diet was 9·0–28·5mg./24hr.

Existing methods for the determination of oxalic acid in blood require comparatively large samples of material (Guillaumin, 1927; Flaschenträger & Müller, 1938; Barber & Gallimore, 1940). Furthermore, most of the existing methods have a relatively poor specificity, although an enzymic method is an exception to this (Crawhall & Watts, 1961; Mayer, Markow & Karp, 1963).

We have recently described a specific fluorimetric procedure for the determination of glyoxylic acid in blood, urine and bacterial extracts (Zarembski & Hodgkinson, 1965). In the present work we have adapted this method to the determination of oxalic acid in blood and other biological materials. Oxalic acid is first separated from other interfering substances by extraction with tri-*n*-butyl phosphate followed by co-precipitation with calcium sulphate. The acid is then reduced to glyoxylic acid in the presence of zinc and hydrochloric acid and coupled with resorcinol to form a coloured fluorescent complex.

EXPERIMENTAL

Reagents. All reagents were prepared in water which was first distilled in glass and then deionized by passage through a mixed-bed ion-exchange resin (Elgastat Deioniser; Elga Products Ltd., Lane End, Bucks.). Tri-*n*-butyl phosphate was purchased from British Drug Houses Ltd. (Poole, Dorset) and L. Light and Co. Ltd. (Colnbrook, Bucks.). Ascorbic acid solution (10%, w/v) was prepared daily. The EDTA reagent was prepared by dissolving 25g. of dipotassium EDTA in 100ml. of 1·8M-potassium carbonate; this reagent was prepared every 2 days and stored at 4°. The remaining reagents were prepared as described by Zarembski & Hodgkinson (1965).

Apparatus. The ultrafiltration apparatus was purchased from Membranfiltergesellschaft, Göttingen, Germany. Samples were shaken in a Griffin Flask Shaker (Griffin and George Ltd., Wembley, Middlesex).

Preparation of zinc spirals. Electrolytic zinc wire, diam. $\frac{1}{8}$ in. (London Zinc Mills, Enfield, Middlesex), was flattened with steel rollers to a thickness of about 2mm. The flattened wire was cleaned with scouring powder, cut into 22cm. lengths, and one end of each piece was wound into a spiral approx. 1cm. long × 1cm. inner diam.

Immediately before use the spiral was placed in silicone fluid M.S. 550, washed under running tap water and dipped several times in freshly prepared 10N-nitric acid until brown fumes appeared. The wire was then washed thoroughly in distilled water and placed for about 5sec. in a test tube containing 2ml. of 1·25N-hydrochloric acid and one drop of methylene blue solution (0·5%). The wire was washed briefly in distilled water and placed for about 5sec. in another tube containing 1·25N-hydrochloric acid. This entire procedure was repeated before each use, one spiral being suitable for approx. ten analyses.

Preparation of samples. Heparinized whole blood or serum (2ml.) was mixed with 0·5ml. of sodium formate (10%, w/v), 0·2ml. of 0·1N-acetic acid and 4·9ml. of water in a 10ml. glass-stoppered tube. The tube was heated in a boiling-water bath for 5min., then 0·4ml. of calcium formate solution (10%, w/v) was added with mixing and heating was continued for another 5min. After cooling, the tube was centrifuged at 1200*g* for 10min. and 6ml. of the supernatant fluid was taken for analysis. Urine was deproteinized if necessary by the same procedure.

Food samples were dried, ground to a fine powder and rendered fat-free (Zarembski & Hodgkinson, 1962). A sample of the fat-free food (2–4g.) was mixed with 8ml. of water, 0·5ml. of formic acid (98%, w/v) and 1ml. of 10N-hydrochloric acid in a 10ml. stoppered tube. After being kept for 30min. at room temperature the contents of the tube were filtered through Whatman no. 1 paper and 2ml. of the filtrate was taken for analysis.

Analytical procedure. The supernatant fluid from serum or whole blood (6ml.) or urine (1ml.+1ml. of water) was mixed with 98% formic acid (0·8ml. for serum and 0·1ml.

for urine) in a 25ml. stoppered tube and 10N-hydrochloric acid was added to make the final strength 1N (0·7ml. for serum and 0·22ml. for urine). Tri-*n*-butyl phosphate (12ml.) was then added. The tube was rocked gently (25–30strokes/min.) for 5min., centrifuged for 5min., and the ester phase was transferred to another 25ml. tube with a Pasteur pipette. Faster shaking during the extraction resulted in the formation of an emulsion which was difficult to break. The extraction of the filtrate prepared from samples of food was performed in a similar way except that the addition of formic acid and hydrochloric acid was omitted, these acids being present already in the filtrate.

The extracted oxalic acid was transferred to an aqueous phase by shaking the tri-*n*-butyl phosphate vigorously with 1·9ml. of 2N-sodium hydroxide for 2min. After centrifuging for 10min. the ester phase was separated with a Pasteur pipette and discarded. Any residual ester was removed by washing with three 5ml. portions of light petroleum (b.p. 40–60°), the last drops of light petroleum being removed by heating briefly in a water bath. After cooling, one drop of bromothymol blue solution was added and the mixture was adjusted to pH7·0±0·2 with dilute acetic acid and sodium hydroxide. The colour was matched with a tris–maleate or citrate–phosphate buffer, pH7·2, containing bromothymol blue.

A saturated solution of calcium sulphate (2ml.) was added, followed by ethanol (13·5ml.), and the contents were mixed gently and allowed to stand at room temperature for at least 3hr. or preferably overnight. After centrifuging for 10min. at 1200*g* the supernatant liquid was removed, the tube drained, and the precipitate was dried for 30min. in an oven at 105–110°.

The precipitate was dissolved in 2ml. of 1·25N-hydrochloric acid and the tube was cooled in ice and shaken gently for several minutes in a mechanical shaker. A freshly prepared spiral of zinc wire was introduced and shaking was continued at 0° for a further 20min. at 8–10 strokes/sec. The spiral was then raised above the solution by bending the top end of the zinc wire over the lip of the tube. The spiral was washed with 1ml. of resorcinol solution (0·5%) and the tube was centrifuged for 3min. at 500*g*.

The dry zinc spiral was removed from the tube, concentrated hydrochloric acid (1·5ml.) was added and the tube heated in a boiling-water bath for 5min. After cooling, 9·6ml. of 1·8M-potassium carbonate was added, followed by 1ml. of 10% (w/v) ascorbic acid solution and 1ml. of EDTA reagent. After 20min. at room temperature the solution was diluted to 25ml. with carbonate–bicarbonate buffer (Zarembski & Hodgkinson, 1965) and, after a further 10min., the fluorescence intensity or the extinction at 490mμ was measured as described by Zarembski & Hodgkinson (1965).

Preparation of standard solutions. A standard solution of oxalic acid dihydrate (55·6μM) in water was prepared freshly every 3 days; this solution contained 5μg. of anhydrous oxalic acid/ml. A series of six tubes was prepared containing 0–10μg. of oxalic acid, the volumes being made up to 2ml. with water. The pH was adjusted to 7·0±0·2, the subsequent treatment being as described previously, although the extraction and precipitation stages can be omitted if desired.

RESULTS

Standard curve. A linear relationship was observed between the amount of oxalic acid in the test solution and the intensity of fluorescence up to 10μg./sample. The relation between extinction and the amount of oxalic acid was linear up to 50μg./sample. In neither case, however, did the calibration line pass through the origin (Fig. 1).

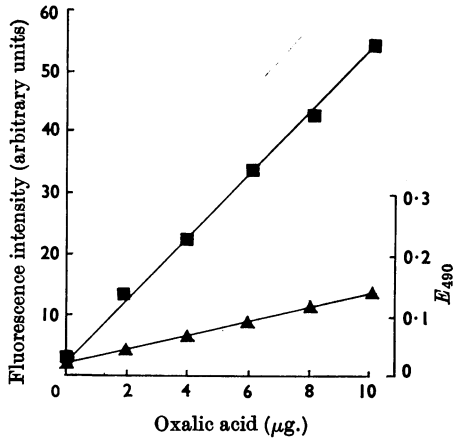

Fig. 1. Relationship of fluorescence intensity (■) and extinction (▲) to oxalic acid concentration (activation wavelength 490mμ; fluorescence wavelength 530mμ).

Table 1. *Effect of* pH *on the recovery of oxalic acid from serum ultrafiltrates*

A sample of normal serum was divided into several parts and HCl or NaOH (2N) was added to give the different pH values listed. After ultrafiltration, the colour was developed in the usual way. Values in columns 2 and 3 are means of duplicate determinations.

pH	Oxalic acid in original serum (μg./2ml.)	Oxalic acid found after addition of 4μg. of oxalic acid (μg./2ml.)	Recovery (%)
5·3	4·2	7·4	80·0
7·0	5·3	9·2	97·5
7·4	4·9	8·7	95·0
8·0	4·7	8·35	91·0

Choice of deproteinizing agent for serum. Deproteinization of serum with acidic reagents (hydrochloric acid, tungstic acid, trichloroacetic acid or perchloric acid) resulted in an incomplete recovery of added oxalic acid. The recovery of oxalic acid after ultrafiltration of serum varied with pH, optimum recovery being obtained at pH7·0 (Table 1). Deproteinization by heating with calcium formate at pH7·0 gave a recovery (97–99%) equal to that obtained after ultrafiltration at pH7·0.

Table 2. *Partition coefficients of oxalic acid in different solvent systems*

Oxalic acid (5mg.) in 2ml. of aqueous solution was shaken with 12ml. of the organic solvent for 5min. After centrifuging for 5min. the two phases were separated and the oxalic acid content of both phases was determined in the usual way.

Solvents	Partition coefficient at 22° $\left(\frac{\text{Concn. in organic phase}}{\text{Concn. in aqueous phase}}\right)$
Butan-1-ol–water	0·18
Isobutanol–water	0·4
Pentan-1-ol–water	0·15
Diethyl ether–water	0·1
Tri-*n*-butyl phosphate–water	3·3
Tri-*n*-butyl phosphate–1N-HCl	9·2
Tri-*n*-butyl phosphate–1N-HCl–1N-formic acid	27·0

Table 3. *Effect of precipitation time on the recovery of microgram quantities of oxalic acid from aqueous ethanol solution*

Water (2ml.) containing oxalic acid in the amounts listed was adjusted to pH7·0 with dilute acetic acid and NaOH. Calcium sulphate and ethanol were added as described under Analytical procedure (Experimental section) and the samples were allowed to stand for 1–16hr. at 22°. The colour was then developed in the usual way.

Oxalic acid added	Oxalic acid recovered (μg.)				
	Time (hr.)...1	2	3	4	16
(μg.)					
2	1·2	1·6	2·0	1·95	2·0
4	3·0	3·8	3·95	4·0	3·9
6	5·4	5·9	6·0	5·9	5·9
8	7·6	7·9	7·8	7·8	8·0
10	9·4	9·9	10·0	9·8	10·0

Deproteinization with calcium formate was chosen since it required less material and a shorter time to perform.

Choice of solvent for the extraction of oxalic acid. The partition of oxalic acid between water and a number of organic solvents was examined. The highest partition coefficient was observed with tri-*n*-butyl phosphate (Table 2). This solvent has the added advantage of low solubility in water. The addition of formic acid and hydrochloric acid to the aqueous phase resulted in a further increase in the partition coefficient. With this system an almost complete recovery of microgram quantities of oxalic acid can be obtained after a single 5min. extraction.

Precipitation of oxalic acid as calcium salt. Preliminary studies indicated that the minimum quantity of oxalic acid that could be recovered quantitatively from aqueous ethanol solution by precipitation with calcium chloride was about 50μg. This was inadequate for the separation of oxalic acid from 1–2ml. of serum since the amount of oxalic acid present is less than 10μg. Improved recoveries were obtained with calcium hydroxide as a precipitating agent but the most satisfactory results were obtained with calcium sulphate.

A quantitative precipitation of less than 10μg. of oxalic acid was obtained when calcium sulphate was added to an aqueous ethanol solution at pH7·0 and the mixture was allowed to stand at room temperature for 16hr. Maximum turbidity of the precipitate was observed when the concentration of ethanol was 70–80% (v/v); a concentration of 75% (v/v) was therefore chosen. Complete recovery of 10–50μg. of oxalic acid was observed within the range pH6·75–7·25, incomplete recoveries occurring at pH6·0 and pH8·0. At 4° complete precipitation of 10μg. of oxalic acid was observed only after 24hr. At 50° the recovery of 10μg. of oxalic acid was incomplete. At 22° under the conditions finally adopted, a quantitative precipitation of 2–10μg. of oxalic acid was obtained in 3hr. (Table 3).

Reduction of oxalic acid to glyoxylic acid. Paget & Berger (1938) reported that, within the concentration range 5–10mg./l., oxalic acid is reduced quantitatively to glyoxylic acid at room temperature in the presence of zinc strip and N-hydrochloric acid. However, under their experimental conditions we could obtain only 45–70% reduction to glyoxylic acid. Estimation of glycollic acid in the reaction mixture by the method of Hodgkinson & Zarembski (1961) indicated that 20–30% of the oxalic acid originally present was reduced to glycollic acid. Further, the extent of reduction of replicate samples of oxalic acid to glyoxylic acid varied from one sample of zinc to another. Complete reduction to glyoxylic acid was achieved in 15min. when electrolytic zinc wire was 'activated' as described, and the reaction was allowed to proceed at 0°. An equimolar quantity of glyoxylic acid, when subjected to the same 'reduction' procedure for 30min., gave an extinction identical with that obtained with oxalic acid.

Reproducibility, specificity and recovery. Sixteen determinations were performed on a standard solution containing 5μg. of oxalic acid/ml.; the standard deviation of these determinations was 0·25μg. (S.E.M. 0·06). The recovery of oxalic acid (5μg.) added to replicate samples of serum (2ml.) was within the range 95–98%. The recovery of oxalic acid (5 and 10μg.) added to urine (1ml.) was within the range 96–99%.

The fluorescence intensity of a number of naturally occurring compounds, structurally or metabolically related to glyoxylic acid, was investigated with 0·1μmole of each compound.

Table 4. *Oxalic acid content of normal urine and daily diets: comparison of different analytical procedures*

Description	Oxalic acid (mg./24hr.) Method 1 (Hodgkinson & Zarembski, 1961)	Method 2 (This paper)
Urine: Subject 1	21·0	20·0
2	15·6	15·0
3	18·0	18·0
4	26·8	26·5
5	27·0	27·0
6	25·0	26·0
	Method 1 (Zarembski & Hodgkinson, 1962)	Method 2 (This paper)
Food: Sample 1	160	165
2	150	160
3	120	125

No detectable fluorescence was observed with glyoxal, sodium pyruvate, α-oxoglutaric acid, α-oxobutyric acid, oxaloacetic acid, formaldehyde, sodium formate, formic acid, glycollic acid, citric acid, malic acid, lactic acid, succinic acid, tartaric acid, malonic acid, acetic acid, salicylic acid or chloral hydrate.

Oxalic acid content of human whole blood, serum and urine. The concentration of oxalic acid in normal non-fasting whole blood from 15 adults ranged from 200 to 320 μg./100ml. and the concentrations in serum varied between 135 and 280 μg./100ml. The daily urinary excretion of oxalic acid by 60 normal adult subjects on a normal diet ranged from 9·0 to 28·5mg./24hr. Good agreement was observed between the values for urinary oxalic acid obtained by the present method and those obtained by the colorimetric procedure described by Hodgkinson & Zarembski (1961) (Table 4). The oxalic acid contents of various foods determined by the present method and by the colorimetric procedure of Zarembski & Hodgkinson (1962) also agreed well (Table 4).

DISCUSSION

In the past the estimation of oxalic acid in blood has been hindered by the lack of an analytical procedure having the necessary sensitivity or specificity. These limitations have been resolved by introducing (1) a new solvent having a high partition coefficient for oxalic acid in aqueous solution, (2) a new precipitation technique which permits the complete recovery of microgram quantities of oxalic acid, (3) a sensitive and specific colour reaction.

Diethyl ether has been most commonly used for the extraction of oxalic acid from biological material. It is possible to obtain a complete recovery of milligram quantities of oxalic acid by continuous extraction with diethyl ether for 6hr. (Hodgkinson & Zarembski, 1961). With tri-*n*-butyl phosphate a quantitative extraction of microgram quantities of oxalic acid can be obtained in 5min. at room temperature. The shorter extraction time and lower temperature help to minimize the conversion of oxalogenic compounds, such as galactose, into oxalic acid (Zarembski & Hodgkinson, 1962). Precipitation of oxalic acid as the calcium salt remains one of the most convenient means of separating the acid from interfering substances. With microgram quantities, however, losses occur owing to the solubility of calcium oxalate in the precipitating medium (Thomsen, 1935). In the presence of carrier calcium sulphate, precipitation of calcium oxalate is complete. The association appears to be relatively stable, since no detectable loss of oxalate was observed when the precipitate was kept in contact with the mother liquor for 2–3 days. The amount of precipitate appeared to be directly related to the amount of oxalic acid present. Recovery of oxalic acid was influenced by the size of the calcium sulphate particles in the precipitate, the recovery being lower with large than with small particles. An excess of SO_4^{2-} ions resulted in a reduced recovery of oxalic acid. These observations suggest that more than one precipitation mechanism may be involved. Samples of zinc available commercially differed considerably in their reducing power, some failing to reduce oxalic acid completely. The over-voltage necessary for complete reduction can be obtained by preliminary treatment of the zinc as described in this paper. Methylene blue was introduced to catalyse the decomposition of any hydrogen peroxide that may be formed; it may also react with zinc in solution to form a double salt and thus maintain the oxidation potential at a constant value (Kolthoff & Sandel, 1952).

Published values of the concentration of oxalic acid in normal human blood show considerable differences, but most recent studies indicate that it is less than 1·0mg./100ml. (Thomsen, 1935; Barber & Gallimore, 1940; Barrett, 1943; Crawhall & Watts, 1961). We have reported that the range of oxalic acid concentrations in normal adult sera is 344–687 μg./100ml. (Zarembski & Hodgkinson, 1963). These values are now considered to be too high, the error being due to the presence of iron in the distilled water. This error has now been avoided by using deionized water. The concentration of oxalic acid in whole blood appears to be higher than that in serum which indicates that the concentration

of this ion in the red cells is higher than that in the plasma. A similar relationship was observed by Flaschenträger & Müller (1938), who reported a concentration of 370 μg./100ml. in whole blood and 210 μg./100ml. in serum.

The present procedure was developed primarily for the estimation of oxalic acid in serum, but it can be readily adapted to other biological materials such as faeces, cerebrospinal fluid and urinary-tract calculi.

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