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RECENT ADVANCES IN MEASUREMENT OF OXALATE IN BIOLOGICAL MATERIALS

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Abstract

Hyperoxaluria is the predominant risk factor in urinary stone disease. A specific, accurate and reliable oxalate assay for urine and plasma is very important for both the diagnosis and efficient management of patients. A review of the advantages and disadvantages of various methods of oxalate determination is presented and is followed by the authors' recommendations.

Key Words: Oxalate, urolithiasis, nephrolithiasis, stone-disease, oxalate oxidase, oxalate decarboxylase.

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Introduction

The pathological significance of oxalic acid in the formation of urinary stones in animals and humans has been known since the early 18th century. Excess consumption of oxalate-rich foods and disturbed metabolism lead to hyperoxaluria which is recognized as a key risk factor for calcium oxalate urolithiasis (Nath et al, 1984; Sharma et al, 1990, 1991). Thus, the knowledge of oxalate concentration in blood and other body fluids can be very important in certain clinical situations such as primary hyperoxaluria (Constable et al, 1979; Scheinman et al, 1984; Pertrarulo et al, 1990), chronic renal failure (Balcke et al, 1982; Chimienti et al, 1986; Wolthers et al, 1986; Borland et al, 1987; Pertrarulo et al, 1990), and calcium oxalate nephrolithiasis (Pinto et al, 1974; Nath et al, 1984; Sharma et al, 1990). Oxalate analysis in blood appears to be extremely useful in the investigation of the reported oxalate transport defect in red blood cells in idiopathic calcium oxalate stone-formers (Baggio et al, 1984; Narula et al, 1989). Also, it will help us to know if in healthy subjects or in patients exhibiting hyperoxalemia the oxalate concentration in whole blood is higher than in serum.

Since the beginning of this century various methods have been formulated for the assay of oxalate from various sources. The majority of the earlier precipitation methods using calcium, lead or other heavy metals, have failed to become generally accepted because of incomplete precipitation. This led to the extraction of oxalate from the samples either with ether or tri-n-butyl phosphate prior to its estimation. Ion exchange chromatography has also been used extensively for the separation of oxalate from various sources and has been shown to yield excellent results. However, the method is more laborious and cannot be applied to a large number of samples. Thin layer chromatographic procedures, using the silica gel G or cellulose plates have been described for successful separation of oxalate. The lack of simplicity is a major draw-back and none has proved ideal. Interference by urinary ascorbate in both enzymatic and non-

enzymatic methods is another major impediment to the development of a simple assay for urinary oxalate. The conversion of ascorbate to oxalate is thought to be catalysed by nitrite and Fe^{3+} at alkaline pH. Since several promising methods have used alkaline conditions, grave errors could be introduced by ascorbate in urine. Suggested methods for eliminating ascorbate interference include pretreatment of the urine sample with ferric chloride, sodium nitrite, ion exchange resins, charcoal, alumina, boric acid or ascorbate oxidase and selective precipitation of oxalate as its calcium salt, and sodium periodate (Mazzachi et al, 1984; Chalmers et al, 1985; Crawford et al, 1985; Rofe et al, 1985; Barlow, 1987; Berckmans and Boer, 1988; Li and Madappally, 1989). Fortunately, methods for preventing these conversions have recently been found. In the presence of oxygen, nitrite catalyses the oxidation of ascorbate to dehydroascorbate which is stable in acid but not in alkaline conditions. Ferric chloride also prevents conversion of ascorbate to oxalate. Perhaps iron forms a stable complex with an intermediate diketogulonate.

Oxalate methodology is not sufficiently advanced to assign a reference method also, there has been no quality control study to check the reliability of urinary oxalate assays. However, Samuell et al (1989) did carry out an external quality assessment of urinary oxalate analysis performed in various laboratories. This survey has yielded some significant findings which will be reviewed and the methods of oxalate analysis will be evaluated.

Colorimetric methods

The most widely used colorimetric method for estimation of oxalate is also employed in our laboratory (described by Hodgkinson and Williams, 1972). Oxalate is co-precipitated with calcium sulphate and ethanol, reduced to glycolate by zinc and sulphuric acid and measured spectrophotometrically with chromatropic acid. Oxalate in human urine was determined by a modification of Hodgkinson's method: precipitation with Ca^{2+}, reduction by activated Zn wire in H_2SO_4 at 190-195°C for 15 min and colorimetric determination of the resulting glycolic acid by chromotropic acid in H_2SO_4 solution at 570 nm (Rozental et al, 1982). Methods have been developed for estimation of oxalate by measuring the glyoxylate formed through its reaction with phenylhydrazine and ferricyanide or by measuring the coloured complex formed between formaldehyde and chromatropic acid. However, the reduction of oxalate in these procedures is critical and if incomplete often results in low recoveries.

Gas liquid chromatography

In the past few years many workers have developed gas liquid chromatographic procedures using a flame ionization detector (Farrington and Chalmers, 1979; Offner and Uring, 1979; Park and Gregory, 1980; Yanagawa et al, 1983). Usually ethyl or methyl esters of oxalate are prepared and separated on a diethyl glycol succinate (DEGS) column. These methods have yielded high recoveries (95-98%). A more sensitive procedure using only 0.1 ml urine sample for oxalate determination was described by Dosch (1979) using a glass capillary column. For blood oxalate analysis, oxalate is extracted from clarified serum, ethylated and analysed by gas chromatography (Nuret and Offner, 1978; Gelot et al, 1980), with recovery greater than 80%. Normal subjects were found to have blood oxalate levels of 27 µmol/L or less. Another gas chromatographic procedure described by Pudich (1983) was found to be suitable for routine clinical determination of oxalate in urine and blood. Although the gas chromatographic method is economical of sample size,it requires special equipment and prolonged column preparation.

Isotachophoresis

Analytical isotachophoresis has been successfully applied to the analysis of organic acids extracted from human urine. Various ion species move at specific speeds in an electric field towards the anode with a leading electrolyte whose mobility is highest and a terminating electrolyte, forming various zones when they reach equilibrium. Quantitation is made by UV absorbance or by a specified thermal signal from the zone. The isotachophoretic measurements are relatively simple and yield high recoveries with good sensitivity. Here too, the isotachophoresis apparatus is expensive and requires special expertise for its use (Schmidt et al, 1979; Tschope and Ritz, 1981; Schwendtner et al, 1982).

Ion chromatography

Ion chromatography is a widely used and highly sensitive technique for determination of urinary oxalate (Robertson et al, 1982; Lopez et al, 1985). The main problem concerning this technique is the on column conversion of endogenous ascorbate to oxalate which has largely been suppressed by treatment of urine with Fe^{+3} boric acid or ascorbate oxidase (Menon and Mehle, 1983; Kasidas and Rose, 1985). Oxalic acid was separated from interfering compounds with a solvent generated ion exchange chromatographic system. The method uses a copper electrode amperometric detector which complexes oxalate for the determination of oxalic acid in biological matrices with a lower detection limit of 5 ng (Kok et al, 1984). Singh and Nancollas (1988) determined urinary oxalate by ion chromatography carried on HPLC-AS_3 with a mixture of 2.42×10^{-4} M $NaHCO_3$ and 1.81×10^{-4} M Na_2CO_3 as mobile phase with 102% oxalate recovery. A more rapid and sensitive method for measurement of oxalate in serum is described by Politi et al (1989) in which deproteinized serum is injected into an ion chromatograph equipped with an anion exchange column and a conductivity detector. Mean serum concentrations of oxalate found by this method

were 21.02 µmol/L. Oxalate was measured by ion chromatography in the ultrafiltrate of heparinized plasma from peripheral venous blood using a membrane with a molecular weight cut off (Schwille et al, 1989). The method has a sensitivity of 0.2 µmol/L, with an intra- and inter-assay coefficient of variation 4 and 12% respectively and an overall oxalate recovery of 100.7%. This method allows the reliable assessment of ultrafiltrable plasma oxalate in health and disease states and in renal calcium urolithiasis. These techniques may help to elucidate oxalate pathophysiology especially the mode of renal handling of oxalate. A simple and rapid procedure for analysis of plasma oxalate in hyperoxaluria has been discussed by Petrarulo et al (1990), in which the ultrafiltered plasma is injected into an ion chromatographic system. The method is highly sensitive (< 1.0 µmol/L) with oxalate recovery of $\sim$95%. It has been used efficiently for analysis of oxalate in samples from patients with primary hyperoxaluria and chronic renal failure.

An ion chromatographic and an enzymatic kit method (Boehringer) for determination of oxalic acid in urine were compared on the basis of reliability and practicality (Classen and Hesse, 1987): both methods gave 100% recovery rate and produced statistically identical results. In terms of cost per analysis ion chromatography is the method currently preferred when a large number of samples is involved.

Isotope dilution and mass spectrometry

Some laboratories have tried various radioisotope techniques for oxalate determination (Hockaday et al, 1965; Gibbs and Watts, 1969; Johansson and Tabova, 1974; Duggan et al, 1979). Quantitative gas chromatography-mass spectrometry (GC-MS) which makes use of stable, isotopically labelled analogues as an internal standard can be used as a reference method as suggested by Koolstra et al (1987). Isotope dilution and mass spectrometry have also been used successfully for the determination of urinary and plasma oxalate (Prenen and Peter, 1983; France et al, 1988). However, because of the requirements of time, labor and instrumentation the use of these methods remains limited.

High performance liquid chromatography (HPLC)

HPLC has enabled excellent separation of fatty acids and other low molecular weight substances from biological fluids. Asper and Schmucki (1979) have proposed an electrochemical detection method for analysing oxalate in urine which does not require pretreatment by HPLC. The combination of detection potential at the carbon paste electrode with a two-buffer-elution technique gives good specificity and acceptable sensitivity for oxalate analysis in urine but not in serum. In other HPLC procedures, oxalate is extracted using tri-n-butyl phosphate.Chromatographic analysis is performed using a fluorescence detector and chromatographic separations are carried out on a 300 x 4 mm octadecylsilane reverse phase column. The fluorescence intensity is measured at 254 nm and the peak area calculated by a data processor. This method is specific, sensitive and readily set up (DiCorcia et al, 1982; Hughes et al, 1982; Sugimoto et al, 1985; Kataoka et al, 1990; Milan et al, 1990).

A procedure based on reverse phase ion-pair HPLC for determination of urinary oxalate was developed by Larsson et al (1982, 1985). Prior to HPLC, urine samples are adjusted to a pH of 2.0 ± 0.1 with orthophosphoric acid. The samples are then injected into an octadecylsilane bonded phase packing. Comparison of this method with others suggests that although HPLC and colorimetric methods do not differ below a level of 400 µmol/L, HPLC is the more precise method and more convenient than the Hodgkinson's procedure. Recently, Fry and Starkey (1991) devised a simple and sensitive assay procedure for measurement of urine and plasma oxalate by HPLC. Acidified urine or plasma samples pretreated with phosphate/neutral acetate buffer are applied to an ion-paired chromatographic system. The method gives good recovery ($\sim$97% with reference range for urinary oxalate excretion = 109-497 µmol/L and plasma oxalate = 0.6-3.9 µmol/L.

Enzymatic determination of oxalate

The draw-backs of the methods evaluated thus far have sustained the quest for simpler, specific and sensitive methods of oxalate determination. The comparative simplicity and specificity of some enzymatic methods appear to fulfill this requirement. The most commonly used enzymes are either oxalate oxidase or oxalate decarboxylase from different sources.

Oxalate decarboxylase

Oxalate decarboxylase, an important oxalate degrading enzyme has been used by several laboratories for measurement of oxalate in biological materials. The enzyme is expensive commercially, but generally easy to produce (the only concern is % purity). Crawhall and Watts (1961) were the first to use oxalate decarboxylase to determine the oxalate content in human plasma while Mayer et al (1963) first developed an oxalate decarboxylase based urinary oxalate determination. These workers precipitated the calcium oxalate salt, dissolved the salt in potassium citrate buffer and determined the evolved CO_2 by the conventional Warburg technique. The primary disadvantage of this method is the requirement of special equipment and technique. Bennett and coworkers (1979) described a radioenzymatic procedure using isotope dilution in conjunction with oxalate decarboxylase. However, the procedure is very cumbersome. The formate produced by oxalate decarboxylase can also be measured by coupling it with formate dehydrogenase and this double enzyme procedure for oxalate determination in urine and blood is described by several workers (Costello et al, 1976; Hatch et al, 1977; Yriberri and Posen, 1980; Beutlar et al, 1985). Previous work from our laboratory had shown the capacity of guinea pig liver

homogenate to degrade oxalate in vitro suggesting the presence of an active oxalate decarboxylase (Murthy et al, 1981). Further purification of this enzyme will open new vistas in research on metabolism of oxalate in mammals. Serum and whole blood oxalate have been determined using oxalate decarboxylase in a linked reaction with NAD requiring formate dehydrogenase and products of reaction measured spectrophotometrically (Hatch, 1990). Specimens need to be processed promptly as oxalogenesis occurs in whole blood standing at room temperature for a few hours as demonstrated by Kasidas and Rose (1986), Costello and Landwehr (1988). Very recently, oxalate decarboxylase has been purified to homogeneity from crude extract of C.velutipes, a basidiomycetes fungus (Mehta and Datta, 1991). Two forms of enzymes were resolved by chromatofocussing. The two isoenzymes were shown to be related by amino acid composition, peptide mapping and immunological cross-reactivity. This enzyme is found to be coded by a single gene and mRNA of this gene is shown to be induced oxalic acid. Purified enzyme from this fungus showed a single step breakdown of oxalic acid to CO_2 and formic acid in absence of any cofactor requirement. The isolation of a new, useful gene will serve as a tool to degrade oxalic acid in plants where it accumulates and acts as a medium for pathogenesis. Further work in this direction will be useful in establishing a simple and reliable assay procedure for determination of oxalate in blood and urine. Preliminary work from our laboratory has shown the ability of guinea pig liver homogenate to degrade oxalate in vitro suggesting the presence of an active enzyme oxalate decarboxylase. Our findings (unpublished) show that the enzyme requires ATP, CoA, Co^{+2}, Mg^{+2} and Fe^{+3} for its optimal activity. Maximum enzyme activity was observed in 25,000 x g pellet indicating that this enzyme may be localised in the peroxisomes. However, more work needs to be done to confirm this observation. Further studies are in progress to isolate, localise and characterize oxalate decarboxylase from guinea pig liver which will be a great asset in the diagnosis and prognosis of hyperoxaluria.

Oxalate oxidase - Oxalate oxidase which catalyses the reaction oxalate $\longrightarrow$ $2CO_2$ + H_2O_2, is estimated either by measurement of peroxide generation or by measuring CO_2 generation. Several studies have reported the successful use of oxalate oxidase to measure oxalate in biological fluids. Potezny et al (1983) used oxalate oxidase prepared from barley roots and immobilized in a continuous flow system to determine oxalate in urine. The H_2O_2 formed from oxalate is detected by color reaction with peroxidase, 3-methyl-2 benzothiazoline hydrazine and N,N'-dimethyl-alanine. The assay system is accurate (95.9% recovery), sensitive ($\leq$ 5 µmol/L), precise and relatively rapid. We have reported the the isolation and purification of oxalate oxidase from sorghum leaves and its utilization for the estimation of urinary oxalate with high sensitivity (0.01 µmol/sample) and reproducibility (Pundir and coworkers, 1984, 1985). Oxalate oxidase immobilized in nylon coil has been used (Kasidas and Rose, 1985; Liedtke et al, 1989) for oxalate determination. A continuous flow assay using immobilized oxalate oxidase was used to measure the level of oxalate in plasma ultrafiltrate (Kasidas and Rose, 1986). Reduction of the spontaneous generation of oxalate in the samples prior to analysis was achieved by acidification and treatment with sodium nitrite. A method for the determination of plasma oxalate using oxalate oxidase and deproteinized plasma has been described with sensitivity of 6-7 nmol (Kasidas and Rose, 1986). The only limitation of the method is interference by inhibitors of glyoxylate converting enzyme with oxalate oxidase, otherwise the method provides good linearity, precision and reproducibility. Recently, Inamdar et al (1991) from Bhabha Atomic Research Centre, Bombay have used a banana oxalate oxidase-horse radish peroxidase-linked assay for estimation of oxalate. Urine samples treated with ascorbate oxidase or charcoal consistently gave a recovery of oxalate close to 100%, thus, suggesting an effective method for preventing ascorbate interference in oxalate measurement. Pundir (1991) has demonstrated the presence of oxalate oxidase in 15,000 x g supernatants prepared from 10 day-old seedlings of three genotypes of Sorghum vulgare. The enzyme is not inhibited by Na^+ and has maximum activity in the pH range of 4.0 to 5.0. The available enzymatic methods of urinary oxalate analysis employing oxalate oxidase from mosses (Laker et al, 1980), barley seedlings (Strauss et al, 1987), beet stem (Obzansky and Richardson, 1983) and banana peel (Raghvan and Devasagayam, 1985; Inamdar et al, 1986) require pretreatment of urine samples to remove endogenous substances especially Na^+ which would otherwise interfere in the assay. The authors have successfully overcome this problem by using oxalate oxidase isolated from sorghum leaves. Further purification of this enzyme from sorghum hybrid genotype CSH-5 is in progress for use in direct enzymic determination of urinary oxalate.

Very recent methods of oxalate estimation with promising results

i) Direct determination of urinary oxalate by continuous flow method: Goldsack et al (1990) described a continuous flow method for estimation of urinary oxalate, using oxalate oxidase and ascorbate oxidase immobilized on the inner surface of O-alkylated nylon tubes. The method is highly sensitive, accurate, specific and is free from interference by other urinary substances. Immobilized enzyme system is stable for 15 months. This method is suitable for both research and clinical use.

ii) Modified enzyme based colorimetric assay of urinary and plasma oxalate: Recently, Wilson and Liedtke (1991) have modified and validated a colorimetric assay involving the use of oxalate oxidase (immobilized in nylon coil) and peroxidase. The O_2 generated reacts with dyes 3-methyl-2 benzothiazolinone hydrazone and 3-dimethylaminobenzoic acid to form a colored complex which is read at 580 nm. Major advantages of the method include improved sensitivity ($<$ 1 µmol/L), the conditions to avoid ascorbate interference and high recovery (86-87%). Plasma concentration in control subjects was 2.5 µmol/L, similar to concentrations determined by recent gas chromatographic and isotope dilution methods.

iii) Improved urinary oxalate kit (Sigma): Sigma attempted to overcome two major problems of positive interference by ascorbic acid and of variable extraction at different pH values by mixing urine samples with buffered EDTA, then treating with activated charcoal, whereas the older method employed by Sigma (R) used absorption of the oxalate onto alumina and elution with sodium hydroxide. Thus,the assay time was reduced and sensitivity increased (Barlow and Harrison, 1990). This modified urine oxalate kit from Sigma overcomes previously highlighted shortcomings of ascorbate interference (Crider and Curran, 1984; Glick, 1987) and with significantly reduced assay time is a welcomed improvement over its predecessor.

Other methods: In addition to the methods discussed, there are several other methods which have been suggested for determination of oxalate in blood and urine. Measurement of urinary and plasma oxalate by capillary gas chromatography using mainly tert butyldimethylsilyl derivatives has been described by several workers (Wölthers and Hayer, 1982; Lopez et al, 1985; France et al, 1988). An enzymatic stopped flow injection method for determination of oxalate by immobilized decarboxylase linked to formate dehydrogenase was recently proposed (Infantes et al, 1991). This method giving excellent results is applicable to the determination of oxalate in urine without separation of oxalate by precipitation . Levya et al (1990) have suggested atomic absorption (AAS) and UV-VIS absorption spectrophotometric (UVAS) procedure for urinary oxalate measurement. The method neither requires precipitation nor previous extraction of oxalate and is free from the interferences often found with analysis of urine samples. Oxalate concetration in urine samples was found to be 8.1×10^{-4} mol/L measured by UVAS and 6.5×10^{-4} mol/L measured by AAS suggesting that AAS is a more accurate technique since it is more selective than UVAS. Inorganic capillary electrophoresis is a new separation technique used for analysis of various anion constituents of urine (Wildman et al, 1991). The procedure moulds the technique of classical electrophoresis with the separation approach of ion chromatography. Matrices which have been difficult to deal with using ion chromatography have proven amenable to analysis by inorganic capillary electrophoresis.

Conclusions

In spite of so many methods for oxalate measurement we have not yet been able to assign a reference method which should be followed in all laboratories. In March 1985, a scheme was initiated by Dr.A.Rose from the U.K., for examination of important aspects of oxalate determination. Our laboratory also participated in this study and some important findings are summarized below:

i) The scatter of results among laboratories was found to be very wide for all methods with a coefficient of variation exceeding 20%.

ii) Positive interference from urinary ascorbate is a major problem in almost all the methods, but now this problem has been significantly resolved using the modified urinary oxalate kit manufactured by Sigma Chemicals and continuous flow methods for oxalate estimation.

Most of the laboratories have produced reasonably good data, possibly by paying strict attention to internal standardization and recovery. The performance can be further improved by abandoning the precipitation-based methods. Most of the laboratories are now using the improved Sigma(R) Kit procedure and this appears to remain the most popular method for laboratories with a moderate workload. Its high cost may be a financial burden for laboratories with a heavy workload. A recent literature survey shows that HPLC and enzymatic methods are gaining wider acceptance for determination of oxalate in biological fluids. The methodology involved in oxalate analysis has been critically asssessed and compared in some of the recent reviews (Zerwekh et al, 1983; Classen and Hesse, 1987; Costello and Landwehr, 1988). These studies show that all methods have a high coefficient of variation within and between assays and none of the methods has proved to be clearly superior to the others.

The most recent methods for determination of oxalate in blood have been based on enzymatic technique probably of its comparative ease of application and specificity. Either oxalate oxidase or oxalate decarboxylase has been used and reaction products are measured by spectrophotometric (Marguire et al, 1981; Hatch, 1990), colorimetric (Kasidas and Rose, 1986; Borland et al, 1987) or potentiometric (Boer et al, 1984; Parkinson et al, 1985) methods. Gas chromatographic methods limit their use because of the requirement for special equipment and the cumbersome procedure. Mean serum or plasma oxalate concentrations have now been demonstrated to range between 1.25 nd 4.01 µmol/L using a variety of enzymatic methods while in vivo isotope studies show lower mean oxalate values of 1.83 µmol/L. Recent reviews (Parkinson et al, 1985; Kasidas

and Rose, 1986; Costello and Landwehr, 1988) demonstrate that similar values can now be obtained by direct methods and isotope dilution procedures, and the gap between these two assay approaches is narrowing.

In our laboratory we are following the procedure of Hodgkinson and Williams (1972) without any modifications. Our laboratory has been participating in the external quality programme conducted by Dr. A.Rose and his coworkers. The interpretation of the results has revealed that our values of urinary oxalate estimated by the precipitation method were very close to those obtained by the Sigma kit method. Hopefully a similar quality control system for oxalate measurement will be implemented shortly in our country, too.

Table I lists briefly various methods reported in the literature for determination of oxalate in biological samples. We have attempted to provide insight into potential advantages/disadvantages of each method which will be of great help while selecting the most appropriate method for oxalate measurement.

Table I

Brief account of different methods of oxalate measurement

Principle	Advantages/Disadvantages	Reference
1. Colorimetric methods		
i) Oxalate $\xrightarrow{(H)}$ Glyoxylate ↓ Phenylhydrazine, $K_3(FeCN)_6$ → Red colored formazan	- Sensitivity: 1 µg	Zarembski and Hodgkinson (1965)
ii) Oxalate $\xrightarrow{(H)}$ Glycolate ↓ Colored complex with chromotropic acid	- Sensitivity:10 µg - Recovery:85% Drawbacks: -Ca precipitation -Incomplete reduction of oxalate by zinc	Hodgkinson and Williams (1972)
iii) Uranium-pyridylazoresorcinol (red color) ↓ Oxalate → Colorless complex	-Sensitivity: 0-300 µg -Highly specific -Recovery: 98%	Badenhuijsen and Jansen (1975)
iv) Oxalate ↓ Activated zinc, H_2SO_4, 190-195°C → Glycolate ↓ Colored complex with chromotropic acid	-Faster than Hodgkinson's method	Rozental et al (1982)
2. Gas liquid chromatography Method includes extraction of oxalic acid, esterification and final analysis by gas chromatograph		
i) Ethyl esters (Blood oxalate) Column: DEGS	-Sensitivity: 10-100 µM -Recovery: 98%	Nuret and Offner (1978) Offner and Uring (1979) Gelot et al (1980)
ii) Methyl esters (Urine oxalate) Column: DEGS	-Sensitivity: 10 µg -Recovery: 98%	Park and Gregory (1980) Moye et al (1983)
iii) Methyl esters (Urinary oxalate) Glass capillary column	-Very Sensitive -Only 100 µl urine is needed	Dosch (1979)
iv) Tert-butyldimethylsilyl derivatives are mainly used for analysis of plasma oxalate by capillary gas chromatography	-Highly sensitive method -Requires special equipment	Wolthers and Hayer (1982) Lopez et al (1985) France et al (1988)
3. Isotachophoresis Oxalate is measured in samples of untreated urine using different leading electrolytes and standardization procedures	-Detection limit 0.04 mmol/L-good sensitivity -High recovery (95%) -Requires special expertise for use	Schmidt et al (1979) Tschope and Ritz (1981) Schwendtner et al (1982)

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4. Isotope-dilution and mass Spectrometry Method makes use of stable isotopically labelled analogues as internal standard for oxalate measurement	-Results comparable with gas chromatographic and HPLC method -Use of radioisotopes and and requirement of special equipment (gas chromatography, mass spectrometry) limits application of this method	Prenen and Peter (1983) Koolstra et al (1987) France et al (1988)
5. Ion chromatography		
i) Oxalate is eluted at alkaline pH (10.5) from anion exchange column	-Alkali elution causes ascorbate interference	Menon and Mahle (1983)
ii) Urine or deproteinized serum injected into ion chromatograph equiped with anion exchange column. Oxalate analysed through conductivity detector	-Sensitivity upto picomol range in blood/urine -Oxalate values are comparable with other methods	Classen and Hesse (1987) Politi et al (1989) Schwille et al (1989) Petrarulo et al (1990)
6. High performance liquid chromatography Oxalate (blood/urine) extracted using tri-n-butyl phosphate. Chromatographic separation carried out on octadecylsilane reverse phase type column	-High sensitivity and specificity -Readily set up -Involves complex methodology and expensive equipment	Larssen et al (1982, 1985) Sugimoto et al (1985) Milan et al (1990) Fry and Starkey (1991)
7. Enzymatic Methods		
A) Oxalate Decarboxylase Oxalate $\longrightarrow$ Formate + CO_2	-Poor sensitivity	
i) Manometry of CO_2	-90%	Mayer et al (1963)
ii) CO_2 trapping in HCO_3^- buffer, and change in pH is measured by change in color	-Sensitivity:5 µg -Recovery:95% -Enzyme inhibition by SO_4^{-2}, PO_4^{-3} ions	Knowles and Hodgkinson (1972)
iii) CO_2 trapping in HCO_3^- buffer, change in pH is measured by electrometry	-Low sensitivity -More sample is needed -Recovery: 95%	Hallson and Rose (1974)
iv) Radioenzymatic procedure -uses isotope dilution in conjunction with oxalate decarboxylase	-Cumbersome procedure	Bennett et al (1979)
v) (Formate dehydrogenase) Formate $\longrightarrow$ CO_2+NADH + NAD O.D. is measured at 340 nm	-Sensitivity: 0-10 µg -Very expensive -Recovery: 98%	Costello et al (1976) Yriberri and Posen (1980) Beutlar et al (1985) Hatch (1990)
B) Oxalate oxidase Oxalate+O_2 $\longrightarrow$ $2CO_2$+H_2O_2		
i) CO_2 trapping ΔpH is measured	-Sensitivity: 5-20 µg -Recovery: 90%	Kohlbecker and Butz (1981)
ii) H_2O_2+ aminophenazone $\downarrow$ catalase Colored compound	-Sensitivity: 0-5 µM -Recovery: 98%	Bais et al (1980)
Other references		Potezny et al (1983) Kasidas and Rose (1985) Pundir et al (1985) Liedtke et al (1989) Inamdar et al (1989,1991) Pundir (1991)

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8. Urinary oxalate kit Based on spectrophotometric determination of H_2O_2 when oxalate is oxidized by oxalate oxidase	-High sensitivity -Ascorbate interference has been totally overcome in latest methods	Crider and Curran (1984) Glick (1987) Kasidas and Rose (1987) Barlow and Harrison (1990)
Other Methods Continuous stopped flow method Method uses oxalate oxidase and ascorbate oxidase immobilized on inner surface of O-alkylated nylon coil	-Highly specific, accurate and precise -No interference from other urinary substances	Goldsack et al (1990)
Enzymatic colorimetric assay Oxalate $\xrightarrow{\text{oxidase}}$ $2CO_2+H_2O_2$ $\xrightarrow{\text{peroxidase}}$ O_2+H_2O Reacts with dyes 3-methyl-2-benzothiazolinone hydrozone and 3-dimethyl amino benzoic acid to form colored complex	-Improved sensitivity -High recovery: 86-87%	Wilson and Liedtke (1991)
Atomic absorption method Copper as oxinate in organic solution is extracted by oxalate in aqueous solution with which copper forms a compound.	-Does not require extraction and precipitation before analysis -No interference by urinary constituents	Levya et al (1990)
Enzyme stopped flow injection method Oxalate determined by immobilized decarboxylase linked to formate dehydrogenase	-Separation of oxalate precipitation	Infantes et al (1991)
Inorganic capillary electrophoresis Procedure merges the technique of classical electrophoresis with ion chromatography. Migration in capillary electrophoresis is dependent on charge-to-size ratio of each of the analytes.	-Very efficient -Requires short analysis time (~6 min)	Wildman et al (1991)

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Editor's Note: All of the reviewer's concerns were appropriately addressed by text changes, hence there is no Discussion with Reviewers.