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Determination of oxalic acid in urine: a review

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Abstract: Available methods for determination of oxalate in urine are reviewed and classified into seven groups, namely: (1) direct precipitation; (2) solvent liquid–liquid extraction; (3) isotope dilution; (4) chromatographic (gas and HPLC); (5) enzymatic (involving enzymes dissolved or immobilized on reactors or electrodes); (6) flow methods (segmented and non-segmented); (7) other methods. In every case the advantages and disadvantages of the different methods are discussed.

Keywords: *Oxalic acid, clinical, review.*

Introduction

Clinical and pathological studies on primary hyperoxaluria [1], a genetic metabolic disorder which often leads to death, have shown it to be the result of impaired conversion of glyoxalate to glycine. This results in excessive generation and excretion of oxalate in urine. Also, moderate increase is observed in some patients with idiopathic renal calcium stones, particularly in those with calcium oxalate stones and associated hypercalcuria. Gerritsen *et al.* [2] reported a decreased excretion associated with hyperglycemia and hyperglycosuria in a mentally retarded child, while pathological intestinal absorption (hyperabsorption [3], malabsorption [4]) can also be detected through an increased concentration in urine oxalate. Likewise, other diseases detectable by oxalate urinary test are nephrolitiasis [5, 6], steatorrhoca [7], ileal disease [8], ethylene glycol poisoning [9] and E-Ferol toxicity syndrome [10]. Thus the determination of oxalic acid or oxalate ion has attracted the attention of several workers and given rise to development of numerous methods for determining this compound [11].

Hodgkinson reviewed the various analytical procedures reported in the literature up to 1969 for determining oxalic acid in biological materials and classified them into four broad groups: (a) direct precipitation; (b) solvent extraction; (c) isotope dilution and (d) enzymatic methods [12]. This classification can now be expanded by adding chromatographic methods and other methods not directly identifiable with the rest. Hydrodynamic methods (segmented and non-segmented flow methods) are considered separately because of their special features and suitability for routine analysis. Each group is treated separately, showing its most relevant features and emphasizing the advantages and disadvantages involved in its use.

Direct Precipitation

The quantitative precipitation of oxalate as calcium salt, followed by permanganate titration, has attracted the attention of many workers because of its simplicity. The

method is fairly straightforward in aqueous solutions and quantities of oxalic acid as low as 5×10^{-7} mol can be estimated with reasonable accuracy. Urine, however, contains many substances that either inhibit precipitation of calcium oxalate or are carried down with the precipitate and react with permanganate. The former compounds include magnesium, polyphosphates and other polyelectrolytes [13–15], while the latter include uric and citric acids [16, 17].

The procedure described by Archer *et al.* [18] has been widely used as a rapid screening test. The principle behind the method is as follows: urine samples are adjusted to pH 5.0–5.2 by addition of acetic acid or ammonia solution. Calcium chloride solution (2 ml of 0.7 M) are added to each 50-ml sample and the mixtures are allowed to stand for 16 h at room temperature. The precipitated calcium oxalate is separated by centrifugation, the supernatant fluid is decanted and the precipitate is washed with dilute ammonia solution. The washed precipitate is dissolved in 0.5 M sulphuric acid and the solution is titrated with 5×10^{-3}M $KMnO_4$ at 60 to 70°C (1 ml of 5×10^{-3}M $KMnO_4$ is equivalent to 5×10^{-6} mol of anhydrous oxalic acid). This procedure has good reproducibility and recoveries ranging from 90 to 93% after addition of ^{14}C-labeled oxalic acid to urine. Hausman *et al.* [19] also precipitate oxalate directly from urine as calcium salt, but they estimate the precipitated oxalate by a colorimetric reaction with indole reasoning that this reaction is more specific than permanganate titration. These authors reportedly achieved recoveries ranging from 95 to 110% when stable oxalate was added to urine. The titration can also be performed with EDTA and murexide as indicator [20], or with Ce(IV) and nitroferroin as indicator [21]. The conditions for the direct quantitative precipitation of oxalate from urine have been revised by Koch and Strong [22], who recommended that a known amount of sodium oxalate should be added to the urine before precipitation and that the precipitation should be carried out at 4°C for one to three days. The use of wash water saturated with calcium oxalate and preserved with pentachlorophenol was recommended in preference to ammonia because of the lower solubility of calcium oxalate in the former. A radioisotope-determined correction factor to allow for the incomplete precipitation of calcium oxalate by interfering substances has been introduced by Mayer *et al.* [23], who used the enzyme oxalate decarboxylase, specific for oxalic acid. The authors applied the procedure of Archer *et al.* [18] for the direct precipitation of oxalate from urine and obtained an average recovery 95% oxalic acid. The use of europium instead of calcium for the direct precipitation of oxalate from urine was recommended by Vittu and Lemahieu [24] because of the low solubility of europium oxalate. Inorganic phosphate is first removed from urine by precipitation as magnesium ammonium phosphate. The solution is then adjusted to pH between 3 and 5, europium nitrate is added and the mixture is allowed to stand for 1 h at room temperature. The recovery of oxalic acid added to urine varies between 88 and 113%, with a mean value of 98%.

Obviously, methods based on precipitation are slow and tedious and completely inadequate for serious cases where the analysis results must be known urgently. In highly refined methods, the delay time is diminished by using correction factors for incomplete precipitation.

Solvent Extraction

Interference by other urinary constituents with precipitation of calcium oxalate can be decreased by prior extraction of the oxalic acid from urine with diethyl ether. However,

the conditions for complete extraction of oxalic acid are critical. Moreover, other organic acids such as citric acid are extracted and may interfere with the subsequent precipitation and titration with permanganate.

The method of Powers and Levatin [16] has been widely used and is carried out as follows: urine (25 ml) is acidified with HCl, heated in a boiling water bath for 30 min and a 10-ml aliquot is extracted with 25 ml of ether for 6 h in a modified Clausen continuous extraction apparatus. Acetic acid (1 ml from a 20 ml l^{-1} solution) is added to the ether extract and the ether is evaporated on a water bath. The aqueous solution is transferred to a conical centrifuge tube, 0.5 ml of dilute calcium chloride solution is added and the mixture is overlaid with 2 ml of dilute acetic acid–alcohol solution. After standing overnight, the sample is centrifuged. The subsequent treatment is similar to that described by Archer *et al.* [18]. The author found that approximately 10% of oxalic acid was lost during the ether extraction, but there was a compensating error, since some calcium citrate precipitated along with calcium oxalate. The amount of calcium citrate precipitated had a titration value equal to about 10% of the calcium oxalate precipitated and was independent of the citric acid concentration.

Numerous modifications of this method have been described. Recovery of oxalic acid has been improved by the use of peroxide-free ether and a more efficient ether extraction apparatus [25]. However, much more rapid and efficient extraction can be achieved by using tri-*n*-butyl phosphate rather than ether. With this solvent it is possible to obtain a quantitative extraction of microgram quantities of oxalic acid in 5 min at room temperature [26]. Improvements in specificity have also been achieved, but this has usually resulted in procedures of greater complexity. Thus, Dodds and Gallimore [27] esterified and distilled the oxalic acid before ether extraction and precipitation as the calcium salt. Specificity has also been increased by replacing the permanganate titration with various colorimetric procedures [26, 28]. Several authors have advocated the preliminary heating of acidified urine to hydrolyse oxaluric acid to oxalic acid [16, 24, 28, 29], however, urine contains only very small amounts of oxaluric acid [30]; moreover, acid hydrolysis causes partial conversion of several urinary constituents to oxalic acid and is therefore likely to cause anomalously high results [31].

Thus, solvent extraction procedures represent developments in the determination of this analyte in urine, though they do not solve the problem altogether.

Isotope Dilution

The use of ^{14}C-labeled oxalic acid to check the recovery of oxalic acid precipitated from urine was mentioned above [18, 22]. Some workers avoid the problems involved in quantitative extraction and precipitation by using the principle of isotope dilution. Hockaday *et al.* [32] added ^{14}C-labeled oxalic acid to a 50-ml aliquot of urine and then precipitated the oxalic acid as calcium salt by the procedure described by Archer *et al.* [18], except that the period of precipitation was overnight at 4°C. The oxalate in the washed precipitate was reduced to glycolate with zinc and sulphuric acid and glycolate isolated by passing the mixture through a Dowex-1-(X8)-acetate column. Aliquots from the column were taken for ^{14}C counting and for the determination of glycolate by reaction with 2,7-dihydroxynaphthalene [33, 34]. The oxalic acid present in the urine is calculated from the specific activity of the isolated glycolate and the total counts of ^{14}C-oxalate added to the specimen by the standard isotope dilution formula. The recovery of oxalic acid added to urine averages 101% (99–103%).

Other isotope dilution methods have been described by Dean and Griffin [35] and Gibbs and Watts [36]. The former authors isolate ^{14}C-labeled oxalic acid from urine by continuous extraction with peroxide-free ether and precipitated the extracted oxalic acid as the calcium salt, the precipitation being repeated several times until the specific activity attained constancy. The stable oxalic acid in the precipitates was determined by the method of Hodgkinson and Zarembski [31]. The procedure of Gibbs and Watts [36] is similar to this [35], except that tri-*n*-butyl phosphate instead of ether is used for the extraction [26]. The colorimetric method proposed by Neas and Guyon [37] has been used in conjunction with isotope dilution by Prenen *et al.* [38] with good results.

Although methods involving isotope dilution enhance the accuracy of the determination compared with non-radioactive methods, they are also rather slower.

Chromatographic Methods

The use of chromatographic techniques has substantially improved determination methods for oxalate as regards selectivity and, in most cases, speed of analysis. Although gas chromatography has provided a larger number of methods, the different modes of high-performance liquid chromatography (HPLC) (normal, reversed-phase and ion chromatography) has been the basis for a pleiad of very interesting methods. (a) The use of gas chromatography sometimes involves laborious esterification processes prior to chromatography. The generic procedure is as follows: a 5-ml urine sample is transferred to a 50-ml round bottom flask and evaporated to dryness using a vacuum rotary evaporator on a waterbath at 45°C. Then, 5 ml 7% HCl–methanol 1 : 4 and 10 μl caprylic acid–methanol solution as an internal standard are added to the residue, which is heated at 60°C for 1 h. The reaction mixture is then transferred to a 10-ml centrifuge tube and 0.5 ml chloroform and 5 ml water are added. After vigorous shaking, the sample is centrifuged at 3000 rpm for 3 min to separate the chloroform layer completely. The upper layer is removed and a 10-μl aliquot of chloroform solution is used for gas chromatography, the eluate being monitored with a flame ionization detector. The temperature of the column is 90°C. The method, proposed by Yanagawa *et al.* [39], features a recovery of 99% and the results are obtained after 2–3 h. The validity of this method has been tested on a large group of individuals. The esterification process may be carried out with methanol [40–42], ethanol [43, 44] or isopropyl alcohol [45]. Other derivatization processes have been developed with the aid of diazomethane in diethylether [46, 47], BCl_3/2-chloroethanol [48, 49], BCl_3/methanol [50], *o*-phenylenediamine/HCl [51], trifluoro bis(trimethylsilyl)acetamide and bromotrimethylsilane [52] or other silanizing agents [53]. The detector most commonly used is flame ionization and, in a lesser proportion, electron-capture [48] and mass spectrometry [44, 45]. Prior to the derivatization process, some workers include a separation step by precipitation [38, 54–56], which obviously slows down the determination, or an ion-exchange process (less slow) [50, 53, 57, 58]. Occasionally the determination can be carried out in about 15 min [53]. Many of these methods have been checked by isotope dilution [46, 54, 55, 57].

Gas chromatography has also been used to study the factors affecting measurements of urinary oxalate [59].

This technique speeds up the determination of oxalate in some cases, nevertheless, a prior separation step which controls the sampling-rate is necessary to obtain completely reliable results. Also the derivatization step is slow in some methods.

(b) High-performance liquid chromatography has fostered the development of methods clearly improving earlier alternatives, although this technique requires relatively expensive instrumentation. These methods can be divided into three groups: (i) methods which include a separation step prior to the chromatographic process; (ii) on-line pre- or post-column derivatization methods; and (iii) ion-chromatographic methods.

(i) The separation step involves the precipitation of the analyte as the calcium salt, an aliquot from the redissolved precipitate is chromatographed, the eluate being monitored by an amperometric detector. The analyte recovery is 97.8%, and the coefficient of variation 2.5% [60]. The limiting step, precipitation, involves incubation time of 30 min. A faster prior separation process involves the use of a Sep-Pak C18 cartridge [61] or a liquid–liquid extraction process [62].

(ii) Derivatization procedures are a means of improving the selectivity of a method without detracting from its speed. Post-column derivatization has been proposed by Ebisuno *et al.* [63], who converted oxalic acid to the hydrazide by reaction with 2-nitrophenylhydrazide in the presence of 3-(dimethylaminopropyl)-1-ethylcarbodi-imide, the coloured product being monitored at 530 nm. The calibration curve has a narrow linear range (2×10^{-4}–1×10^{-3}M), the average recovery being 101%.

Pre-column derivatization has been performed with the aid of *o*-phenylenediamine [64–66] or 9-anthyldiazomethane [62] (photometric and fluorimetric detection respectively), with normal [64] or reversed-phase [62, 65, 66] chromatography. The formation of a coloured compound requires drastic conditions (concentrated HCl and a temperature of 130°C for at least 15 min); to obtain the fluorescence ester, the reaction mixture is required to be kept at room temperature for 90°C.

(iii) Ion-chromatography with conductimetric [67–70] or amperometric [71] detection has provided a series of methods with detection limits between 8.3×10^{-6}M [67] and 5.5×10^{-8}M [71], with or without prior additional separation step (precipitation with $BaCl_2$) [67] or ion-exchange [69], which in general result in good reproducibility and recovery, except in the method proposed by Toyoda [67], the recovery in which was only 84%.

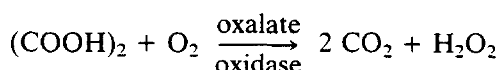
The most serious shortcoming of the methodologies based on liquid chromatographic processes is the instrumentation required, which is expensive for modest clinical laboratories.

Enzymatic Methods

There are two types of enzymatic processes which form the basis of selective measurement of the oxalate concentration in biological fluids: catalysis by oxalate oxidase or oxalate decarboxylase, each of which has allowed the development of a similar number of methods. Most existing methods make use of these catalysts in solution; nevertheless, there is a growing trend towards immobilization of these substances on a suitable support, the immobilized enzyme making up a reactor or electrode. Enzymatic methods for this analyte have reached such a degree of development that commercial kits are now available [72–74].

Only batch-enzymatic methods are discussed in this section because flow-enzymatic methods are included in the following section.

(a) *Use of oxalate oxidase.* Oxalic acid is oxidized by atmospheric oxygen in the presence of this enzyme yielding carbon dioxide and hydrogen peroxide according to the reaction:



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

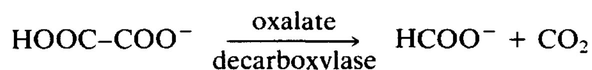
the monitoring of which can be carried out through any of the products.

The straightforward method, proposed by Kohbecker *et al.* [73], is based on the change in pH caused by the absorption of released carbon dioxide into a buffered alkaline solution. The determinative range is 8×10^{-6}–1.6×10^{-3}M.

Other methodologies make use of a coupled reaction in which the hydrogen peroxide formed reacts (aided by peroxidase) with an organic compound to yield a coloured product which is used to monitor the overall reaction [75–84]. An example of this is the method proposed by Buttery *et al.* [82], who use the following procedure: to 1.9 ml of distilled water in plastic tubes (12 × 85 nm) add 0.1 ml of water (blank), urine or standards. Then add 0.1 ml of 5×10^{-3}M NaOH, mix and add one spatula point of charcoal (approximately 10 mg) only to the tubes containing urine. Vortex-mix and centrifuge for 10 min the tubes containing charcoal. Into 12 × 100 mm glass tubes, place 0.1 ml of blank, supernatant (in duplicate) or standards. Add 2.0 ml of freshly prepared colour reagent containing 0.2 ml of peroxidase and 0.5 ml of oxalate solution in 30 ml of citrate buffer to all tubes mix and leave the tubes in the dark at room temperature for 2 h. Measure the absorbance of the samples at 595 nm versus the reagent blank and determine oxalate concentration in urine by comparison with the standard curve.

A generic problem involved in the use of oxalate oxidase is the inhibition of its activity by the presence of divalent cations and anions such as fluoride and iodacetate. This interference can be minimized by sample pretreatment with charcoal [70, 82], in addition to suitable dilution (at least 20-fold). Ascorbate has a positive interference very difficult to eliminate so far [81]; yet, the interference from ascorbic acid can be eliminated by treating the acidified sample with $FECl_3$. The incubation time is dramatically shortened in some methods (60 min [82], 30 min [79], 15–20 min [87]) or lengthened if a prior separation (precipitation) step is involved [83]. The determination ranges typically obtained are between 1×10^{-5} and 5×10^{-5}M, while recoveries are generally in the range 90–110%.

(b) *Use of oxalate decarboxylase.* This enzyme acts on oxalate ion by decomposing it into formate and carbon dioxide, according to:



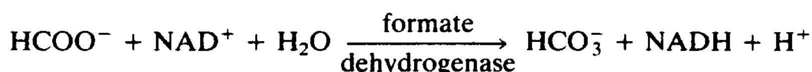
$$HOOC\text{–}COO^- \xrightarrow[\text{decarboxylase}]{\text{oxalate}} HCOO^- + CO_2$$

which allows direct measurement of the product, CO_2, or the use of an additional enzymatic reaction involving formate.

The direct monitoring of the CO_2 released has been performed by Sallis *et al.* [84], who used an apparatus designed by themselves to trap carbon dioxide, which was absorbed into a continuously circulating $Sr(OH)_2$ solution. The linear determination range is 1×10^{-7}–3×10^{-6}M. The conductimetric method proposed by Bishop [85], similar to this, involves prior extraction with chloroform and subsequent incubation of the solution with enzyme for 16 h. In the Hallson and Rose method, carbon dioxide is collected on a basic buffered solution, whose change in pH or colour (use of phenolphthalein) is related to the oxalate concentration in the urine [86] or plasma [87] sample. An incubation period of 18 h at 37°C is required. The enzymatic reaction is inhibited by the presence of

sulphate and phosphate, but this adverse effect is overcome by using a suitable enzyme concentration (above 0.15 U ml^{-1}).

The use of coupled enzymatic reactions involves the following step:



$$HCOO^- + NAD^+ + H_2O \xrightarrow[\text{dehydrogenase}]{\text{formate}} HCO_3^- + NADH + H^+$$

The method developed by Costello *et al.* [88] uses this reaction coupled to decarboxylation and involves monitoring of the reduced coenzyme at 340 nm. The practical procedure is as follows: aliquots of fresh 24-h urine (200 μl) or urine extracts (100 μl) are added to cuvettes containing citrate buffer (0.1 M, pH 3.0), 0.25 ml and EDTA (0.05 M) 0.1 ml. The reaction is started by addition of oxalate decarboxylase, 10 μl (0.04 U) and allowed to proceed for 30 min at 37°C. At this stage, 1.6 ml of phosphate buffer (0.5 M, pH 7.0) containing NAD^+ (2.4×10^{-6} mol) and 20 μl of formate dehydrogenase (0.16 U) are added, and the absorbance change is monitored for 6–10 min, depending on the analyte concentration. Oxalate recovery solutions, aqueous standards and blanks are similarly prepared. The method is not too sensitive (linear range of the calibration curve between 1×10^{-5} and 2×10^{-4}M with recoveries between 95 and 103%, tested by a radiometric method.

The inhibitor effect of ascorbic acid on oxalate decarboxylase has been studied by some authors, who recommend the use of ^{14}C-labeled oxalate [89, 90] to eliminate this disturbance or even a prior separation process of the analyte by precipitation as calcium salt [91, 92].

One rate-analysis method involves re-dissolution of the calcium oxalate precipitate, addition of the enzymes in citrate buffer of pH 3 and incubation for 1 h, halting of the reaction by addition of phosphate buffer of pH 7.8, and monitoring of the absorbance at 340 nm [93]. A recovery of 95% is achieved, with a detection limit of 2×10^{-5}M and a coefficient of variation smaller than 9.5%. The results provided by this method are consistent with those obtained by gas chromatography and an equilibrium enzymatic method, with the added advantages of kinetic methods as regards sensitivity and rapidity.

Automatic batch analysers have been suited to enzymatic methodologies for determination of oxalate. Thus, the method proposed by Urdal [94] uses a COBAS BIO analyser and is based on the two enzymatic reactions mentioned above (aided by oxalate decarboxylase and formate dehydrogenase), with photometric monitoring of the NADH formed at 340 nm, the linear range of the calibration curve extending down to 2.5×10^{-3}M, with a detection limit of 2.5×10^{-5}M. The method has good correlation (r = 0.95) with measurements performed after precipitating the calcium oxalate in ethanol; thus, it can be applied directly to unprocessed urine samples. This centrifugal analyser allows up to 12 samples to be analysed per hour.

A comparison of enzymatic and ion-chromatographic determination of urinary oxalate has been performed by Classen and Hesse [74]. For ion-chromatographic determination, a Dionex column was used. For the enzymatic assay, a Boehringer–Mannheim 7555699 test kit was used for urine samples (20 ml, initial pH 1) which has been heated at 80°C for 15 min with EDTA (10 mg), cooled and adjusted to pH 5. Ascorbic acid, which interferes at 6×10^{-4}M, was oxidized with an ascorbate oxidase spatula. Although the coefficient of variation (N = 20) for oxalate was lower by chromatography (1.3–1.9%) than by enzymatic assay (2.2–4.9%) the methods gave similar results and the enzymatic assay is proposed as an alternative to the chromatographic procedure.

Considering the methods commented on above, enzymatic methods seem to be faster and easier to implement; nevertheless, they have a serious shortcoming arising from the cost of enzymes. This negative aspect can be minimized by using enzymes physically, chemically or physico-chemically bound to a suitable support [95, 96] and making up enzyme reactor or electrodes.

(c) *Use of enzyme electrodes*. Kobos and Ramsey have designed an enzyme electrode [97] using oxalate decarboxylase immobilized on a carbon dioxide gas-sensing electrode. The system has a linear response with the logarithm of the oxalate concentration between 2×10^{-4} and 1×10^{-2}M, and is not affected by the presence of phosphate or sulphate at the normal levels found in urine. The electrode response is stable after one month work.

Oxalate decarboxylase has been immobilized on the tip of an oxygen (Clark) electrode, which controls the partial pressure of oxygen in the sample, a parameter which changes as a function of the development of the enzymatic process. The method is sensitive (the linear range being 1.3×10^{-5}–2.2×10^{-4}M) and is endowed with good stability, selectivity and precision [98]; also, it allows the use of unprocessed urine. The enzyme electrode response to oxalate in urine samples from 14 patients compared favourably with that obtained by the standard spectrophotometric method.

A comparative study of electrodes using oxalate oxidase immobilized on a carbon dioxide gas-sensing electrode and on a hydrogen peroxide sensor probe has been performed by Fonnong [99]. The amperometric method has a lower determination range (2×10^{-5}–3×10^{-4}M versus 1×10^{-4}–2×10^{-3}M for potentiometric one), better recovery (98 versus 96%) and a slightly smaller relative standard deviation (3.6 versus 3.8%). Sample pretreatment involves the use of an anion-exchange column (Chelex-100) after heating at 60°C for 15 min.

The most serious shortcoming of these methods is the electrode construction, they have to be home made because no probes of this type are commercially available.

Continuous Methods

Both non-enzymatic and enzymatic segmented and non-segmented flow methods have been reported. Enzymatic methods use oxalate oxidase or oxalate decarboxylase almost exclusively immobilized and the auxiliary enzyme, if required for the coupled reaction, in solution.

A Technicon Autoanalyser has been used for the determination of oxalate with oxalate oxidase immobilized on nylon [100–102] and with the aid of peroxidase to yield the coloured monitored compound. In the method proposed by Bais *et al.* [101] the monitoring of the reaction product of the 4-aminoantypyrine/phenol/hydrogen peroxide reaction at 520 nm is interfered by the presence of ascorbate and NADH in the medium; in the method proposed by Potezny *et al.* [100], the product of the 3-methylbenzothyazolin-2-one hydrazone/*N,N*-dimethylaniline/hydrogen peroxide reaction, monitored at 600 nm, is only disturbed by ascorbate at non-physiological concentrations, therefore allowing up to 10 analysis to be carried out per hour. The method of Kasidas and Rose [102] eliminates the interference from ascorbate by treating urine with sodium nitrite; it affords a recovery of 93%, and a coefficient of variation of 5%. The sampling frequency is 15 h^{-1} and the results correlate well (r = 99) with those obtained with oxalate decarboxylase.

The continuous segmented flow methods using dissolved oxalate dehydrogenase are

based on measurements of the CO_2 generated and phenolphthalein. The inhibition caused by sulphate and phosphate ions is compensated for by addition of these anions to the standards. The detection limit achieved is 5×10^{-5}M and the standard deviation 3.5% for ultrafiltrated samples [103]. The rest of the methods use a coupled reaction in the presence of formate dehydrogenase and NAD^+, monitoring the absorbance of the reduced coenzyme after a separation step [104, 105]. The use of dissolved enzymes in flow methods raises the cost per analysis to $1.5.

A non-enzymatic segmented flow method uses a Technicon instrument for indirect determination through calcium from the re-dissolved precipitate of calcium oxalate, which reacts with cresolphthalein complexone [106].

Several non-segmented flow injection (FIA) [107] methods using non-enzymatic and enzymatic reactions have been proposed. The Gaetani *et al.* method [108] uses the oxalate isolated from acidified urine samples by precipitation with $CaCl_2$ after dissolution. Portions (10 μl) of the diluted solution are injected into a carrier stream of 0.35 M H_2SO_4 which is mixed with a solution of zirconyl chloride (3×10^{-6}M)/flavonol (1.47×10^{-6}M). The fluorescence quenching produced by the oxalate is measured at 480 nm (excitation at 350 nm). The calibration graph (peak area versus oxalate concentration) is linear from 1×10^{-5} to 3.6×10^{-4}M and the detection limit achieved is 6×10^{-6}M. Recovery is 95% and the coefficient of variation is 7%. The results obtained agree well with those found by conventional methods. A single-channel FIA configuration has been proposed to automate the method described by Dutt and Mottola [109] based on the promoting effect of oxalate on the reaction between ferroin and Cr(VI). A similar manifold is required for the method based on the inhibitory effect of oxalate on the 2,4-diaminophenol/hydrogen peroxide system, catalysed by Fe(III) [110], which is carried out by normal FIA and stopped-flow/FIA. The typically kinetic character of the measurements in the last case makes unnecessary the pretreatment of the urine samples. The sampling frequency is 20 h^{-1} [111].

Only one enzymatic-FIA method making use of immobilized oxalate decarboxylase on a controlled-pore glass reactor and dissolved formate dehydrogenase has been proposed so far. The kinetic-photometric monitoring of the reduced coenzyme makes the determination more selective. The doubly stopped-flow configuration [112] used allows stopping of the injected sample in the reactor for the time needed to achieve optimum conversion of oxalate into formate; then, the sample plug is circulated again and merged with a formate dehydrogenase stream. The mixture of the two streams is stopped in the detector, where the evolution of the last reaction is monitored. A beam of calibration curves is obtained, their sensitivity (slope) depending on the stop-time at the detector (1–4 min) and the linear range extending from 1×10^{-4}M to 2.0×10^{-2}M, coefficient of variation 2%. The recovery of the added oxalate and the comparison of the results from processed (precipitation as calcium oxalate) and unprocessed urine samples, shows the excellent features of the method, which allows up to 20 analyses to be performed per hour [113].

An unusual unsegmented flow method makes use of oxalate oxidase immobilized on glutaraldehyde-activated controlled-pore glass. The product is packed in a 1-ml plastic column, which is mounted on an enzyme thermistor unit (an insulated thermostatically controlled aluminium block in which small temperature increases are measured and recorded). A citric buffer (pH 3.5) containing 8×10^{-4}M quinolin-8-ol and 2×10^{-3}M EDTA is pumped through the column. The sample solution (0.5 ml) is injected into the buffer stream via a six-way valve, and the temperature increase is recorded (2 min are

usually sufficient for each sample). The curvilinear calibration graph permits the determination of 1×10^{-5}–11×10^{-3}M oxalic acid. Urine samples are analysed directly for 2×10^{-4}M oxalic acid; below this level, an extraction step with tributyl phosphate is required to overcome the inhibitory effect of other anions [114].

Other Methods

In this group are included a series of very different methods not so numerous as to deserve a separate discussion.

Isotachophoresis has been applied to unprocessed urine samples at an analysis time between 20 min and 1 h [115]; the photometric monitoring at 254 nm provides a determinative range between 1×10^{-4} and 2×10^{-3}M. The recovery of added oxalate (1×10^{-3} mol) ranges between 70 and 100% and increases to 80–110% by the use of 1×10^{-2}M $ZnCl_2$ as electrolyte. When isotachophoresis is carried out on samples which from oxalate has been previously separated as calcium salt, separation is complete within 40 min; thereby eliminating the interference of other acids present in urine and yielding improved results [116].

Cyclic voltammetry and carbon electrodes (paste and vitreous) have been used in conjunction for determination of oxalate in urine, associated to a chromatograph and, in general, to a hydrodynamic system [117].

Sample clean-up on a cation resin (Chelex-100 or AG50W-X4, 200–400 mesh) has been proposed for measurements of oxalate in urine samples containing ascorbic acid, the oxalate being determined by spectrophotometry at 592 nm by use of 3-methylbenzothyazoli-2-one hydrazone and dimethylaniline as reagents. The inter- and intra-assay coefficient of variation range from 7.0 to 13.2% and from 4.4 to 6.5%, respectively, and the mean recovery is 90%. There is no interference from ascorbic acid or 3,4-hydroxyphenylacetic acid. Use of ascorbate oxidase for oxidation of ascorbic acid is preferable to the use of $FeCl_3$ or charcoal, which give low and high results, respectively, for oxalate [118].

An indirect determination of oxalate can be carried out by precipitation as calcium salt and measurement of calcium, by atomic absorption spectrometry after re-dissolution [119].

Other methods involve prior separation procedures using charcoal, liquid–liquid extraction and formation of mixed coloured complex [120], extraction into tributylphosphate or chloroform and reduction to glyosylic acid or formation of hydrazone [121, 122], or the isolation of oxalate by ion-exchange in the presence of EDTA, reduction of the eluted acid with zinc and colorimetric determination at 570 nm by reaction with chromotropic acid [123] or, even a colorimetric determination based on the decrease in the absorbance of the red U(IV)/4-(2-pyridylazo)resorcinol complex after separation of the analyte by precipitation [114]. The concentration of oxalate in the samples is calculated from an equation derived from recovery experiments involving ^{14}C-labeled oxalic acid.

The determination of this analyte based on reaction-rate promoting effects [125] on the oxidation of ferroin by Cr(VI) in a sulphuric medium has been proposed by Dutt and Mottola and applied to oxalate in urine and serum samples [126]. The initial rate is evaluated from the slope of the reaction curve extrapolated to near-zero reaction time and the amount of oxalic acid is calculated by reference to a working curve. A prior

precipitation/liquid–liquid extraction step is required and the total analysis time is 45–60 min.

Conclusion

Continuous enzymatic methods seem to be most suitable for the determination of oxalate because the possibility of immobilizing the catalyst eliminates the most important shortcoming, namely the high cost of oxalate oxidase or oxalate decarboxylase. Auxiliary enzymes such as formate dehydrogenase (lower cost) used in solution, allow kinetic measurements to be performed, thereby making the methods more selective.

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