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Measurement of Urinary Oxalate: An Enzymatic and an Ion Chromatographic Method Compared

By *A. Classen* and *A. Hesse*

Experimentelle Urologie, Urologische Universitätsklinik Bonn

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Summary: An ion chromatographic and an enzymatic method for determination of oxalic acid in urine were compared on the basis of reliability and practicability. Both methods displayed a within-run imprecision of under 5% and a total imprecision of under 10%. The mean recovery of 0.2 mmol/l sodium oxalate lay close to 100% for both methods. After 80 parallel determinations, both methods produced statistically identical results. In terms of cost per analysis, ion chromatography is the method currently preferred when large numbers of samples are involved. If only a few samples are to be analysed the enzymatic method – without any loss of reliability – can be employed.

Introduction

An investigation conducted by *Vahlensieck* et al. (1), based on a representative poll carried out by the Institute for Applied Social Sciences (INFAS, Bonn-Bad Godesberg), found that the incidence of urolithiasis in the Federal Republic of Germany lay at 0.5%. Assuming that the population numbers 60 millions, this would mean that annually some 300 000 persons suffer from urinary stones. Approximately 65% of these concrements consist of calcium oxalate. This high relative frequency, the reason for which is still unexplained, together with the fact that – unlike other types of stones – these cannot be treated chemolitholytically, make it essential both for reasons of basic research and prevention of relapse, that we should be able to determine the calcium and oxalic acid in the urine of patients suffering from urinary stones. Determining the calcium in urine is no problem, but the analysis of the oxalic acid is more complicated. A review of the numerous methods now available for determining oxalate in urine has been published by *Robertson & Rutherford* (2) and *Gay* et al. (3). Many of these methods are extremely time-consuming and/or have been found to be liable to error. Hence only a few of them are employed routinely in the laboratories concerned. In recent years, so-called "suppressed ion chromatography" has become increasingly popular as a method for determining urinary oxalate (4–8). This involves separating oxalate from the remaining anions on an anion exchanger and then detecting it through the electrical conductivity of its acid. One disadvantage of this method lies in the very high cost of the equipment used, and hence this method is really only suitable for certain research laboratories where an appropriate supply of samples is available. The remaining laboratories must resort to methods more economical. The aim of our investigation was to compare an enzymatic method which has recently been adapted for the determination of oxalate in urine based on the oxalate decarboxylase/formate dehydrogenase enzyme system (9) with the suppressed ion chromatographic method with respect to their practicability and reliability.

Materials and Methods

Methods of determining oxalate

Suppressed ion chromatography

The ion chromatographic analysis was conducted using a Dionex 2000i system (Dionex Deutschland GmbH, Weiterstadt, FRG). This was fitted with a 50 µl sample loop, an NG1

precolumn, an AS4 separator and an AFS1 hollow-fibre suppressor. A high pressure filter unit was installed before the precolumn. The eluent was 2.8 mmol/l $NaHCO_3$ and 2.2 mmol/l Na_2CO_3. The flow rate of the eluent was 1.6 ml/min. The sensitivity of the detector was set at 1 µS full scale. The preparation of the samples was carried out along the lines suggested by *Robertson & Scurr* (8), 0.5 ml 1 mol/l HCl being added to 1 ml of thoroughly mixed urine to which, after a delay of approximately 1 hour, 0.3 mol/l H_3BO_3 was added to make up 50 ml. Injection was effected without prior filtration of the sample. The chromatograms were analysed using a Shimadzu C-R2AX integrator (Shimadzu Europa GmbH, Düsseldorf, FRG), via the peak area, by means of comparison with a calibration curve from aqueous standards at concentrations of 0.050–0.700 mmol/l, which were renewed for each series.

Enzymatic analysis

The test combination used was No. 755 699 from Boehringer Mannheim (Boehringer Mannheim GmbH, Mannheim, FRG), which has been placed on the market for the food laboratories. The preparation of the samples was modified for use in connection with urine, in that 20 ml of the sample were acidified with 0.5 ml HCl (25%) to pH 1 (test strip pH 0-14, Merck, Darmstadt, FRG), approx. 10 mg EDTA added and the whole heated for 15 minutes in a water bath at 80 °C. After cooling to room temperature, 1 ml of a phosphate/citrate buffer (pH 5) containing 0.13 mol/l potassium phosphate and 0.07 mol/l citric acid, was added and the pH was adjusted to between 4 and 6 using 0.35 ml 10 mol/l NaOH and 0–0.35 ml 1 mol/l NaOH. As the manufacturers had indicated that ascorbic acid in concentrations over 0.6 mmol/l would interfere in the reaction, ascorbate was checked semiquantitatively using a test strip (Rapignost, Behringwerke AG, Marburg, FRG) and, when necessary, removed using an ascorbate oxidase spatula (Boehringer Mannheim). Acting in accordance with the manufacturer's instructions, 0.2 ml of the sample prepared as above was now used for enzymatic determination (tab. 1). The concentration in the sample was calculated using the molar lineic absorbance of NADH ($\varepsilon^{NADH}_{334\,nm}$ = 618 m^2/mol) in the following manner: the difference ΔA_{ox} was determined from absorbance ($A_2 - A_1$) of the sample or the standard which was used as a control (0.25 mmol/l) and absorbance of the blank reading. Using a cuvette of 1 cm light path and reading at 334 nm, the analyte concentration may be determined from the following formula:

$$c = 0.0194 \cdot \Delta A_{ox} \cdot F \text{ (mmol/l)}$$

F is the sample dilution factor, which is obtained from the quotient of the final sample volume and the initial sample volume.

Sample material and method comparison experiments

The sample material consisted of 24 h and spontaneous urine from both stone patients and healthy test persons. All samples were taken away for analysis immediately on collection. In order to establish *within-run imprecision*, three urine specimens from the lower, middle and upper concentrations were each analysed 20 times and the coefficient of variation selected as a measure of the imprecision. *Total imprecision* was estimated by analysing three samples of lower, medium and high oxalate concentration over a period of 10 days in duplicate (a, b) and calculating the coefficients of variation from the b values. During the period of the investigation the samples were stored in a deep-freeze (−20 °C). In order to assess the *accuracy* of both methods we conducted recovery experiments on 20 urines, using 0.2 mmol/l of sodium oxalate; both the basic urines and the supplemented samples were each analysed 4 times and recovery was calculated in percent from the mean values obtained. In addition to this, the oxalate in 80 urine samples was determined using both methods of analysis, and double determinations (a, b) were carried out. The evaluation of the non-parametric procedure after *Passing & Bablock* (10, 11) and all the test statistics relating to the regression line were calculated at $p < 0.05$.

Results and Discussion

In the range 0.050–0.700 mmol/l oxalate both methods of analysis displayed a linear relationship between the analyte concentration and the detector response. This range is considered to be perfectly adequate, as in our experience over 95% of all samples can be assessed without further dilution and analysis. *Within-run imprecision* by both methods was good, lying between 1.3 and 1.9% (ion chromatography) and 2.2 and 4.9% (enzymatic method) (tab. 2). *Total imprecision* was found to be between 2.9 and 5.3% for the ion chromatographic and 4.3 and 8.0% for the enzymatic method (tab. 3).

In both methods, the highest coefficient of variation was found at the lowest sample concentrations. The enzymatic method displayed higher coefficients

Tab. 1. Pipetting instructions for the enzymatic determination of oxalic acid.

Pipette into cuvets	Sample blank	Sample	Reagent blank	Standard
Buffer solution (pH 5)	0.10 ml	0.10 ml	0.10 ml	0.10 ml
Urine sample (pH 5)	0.20 ml	0.20 ml	–	–
Standard solution	–	–	–	0.20 ml
Bidest. water	–	–	2.20 ml	–
Oxalate decarboxylase solution	–	0.05 ml	–	0.05 ml
Mix, leave standing for 30 min at room temperatur. Add				
NAD/phosphate buffer	2.00 ml	2.00 ml	2.00 ml	2.00 ml
Bidest. water	0.05 ml	–	0.05 ml	–
Mix, measure absorbance of the solution (A_1) after 2 min. Start reaction by adding				
Formate dehydrogenase	0.05 ml	0.05 ml	0.05 ml	0.05 ml
Mix, leave standing, sealed, for 40 min at room temperature. Measure the absorbance of the solutions directly after each other (A_2)				

throughout, which is most probably attributable to the greater number of manual working stages. The mean *recovery* was 100.9% for the ion chromatographic method and 98.7% for the enzymatic method (tab. 4), with the initial concentration of the supplemented samples found to be between 0.046 and 0.524 mmol/l (ion chromatography) and 0.042 and 0.558 mmol/l (enzymatic). The *parallel determinations* revealed a high level of agreement between both methods (fig. 1). The slope of the regression line, at 0.977, did not vary significantly from 1, and, similarly, the intercept did not vary from 0. Statistically speaking, this means that the methods produced identical results. We attribute this encouraging result to the fact that both these analytical processes eliminate three cardinal sources of error inherent in other methods i. e. quantitative errors through

a) preformed calcium oxalate crystals,
b) interference from ascorbic acid, and
c) losses during separation of oxalic acid.

Tab. 2. Within-run imprecision.
Abbreviations/symbols:
x̄ mean,
s standard deviation,
CV (%) variation coefficient in per cent,
min minimum value,
max maximum value,
n sample size;
concentration data in mmol/l

Samples	Statistics	Method	
		Chromatographic	enzymatic
1	x̄	0.095	0.100
	CV (%)	1.8	4.9
	min	0.092	0.092
	max	0.098	0.109
	n	20	20
2	x̄	0.276	0.269
	CV (%)	1.3	2.2
	min	0.271	0.256
	max	0.281	0.279
	n	20	20
3	x̄	0.542	0.552
	CV (%)	1.9	2.9
	min	0.525	0.517
	max	0.562	0.585
	n	20	20

Tab. 3. Total imprecision.
Abbreviations/symbols:
x̄ mean,
s standard deviation,
CV (%) variation coefficient in per cent,
min minimum value,
max maximum value,
n sample size;
concentration data in mmol/l

Samples	Statistics	Method	
		chromatographic	enzymatic
1	x̄	0.063	0.062
	CV (%)	5.3	8.0
	min	0.055	0.059
	max	0.068	0.060
	n	10	10
2	x̄	0.114	0.110
	CV (%)	4.5	5.1
	min	0.100	0.105
	max	0.120	0.122
	n	10	10
3	x̄	0.543	0.520
	CV (%)	2.9	4.3
	min	0.511	0.490
	max	0.563	0.549
	n	10	10

Tab. 4. Recovery.
Abbreviations/symbols:
x̄ mean,
CV (%) variation coefficient,
min minimum value,
max maximum value,
n sample size;
recovery in per cent

Statistics	Method	
	chromatographic	enzymatic
x̄	100.9	98.7
CV (%)	4.5	5.2
min	93.5	90.5
max	108.5	106.5
n	20	20

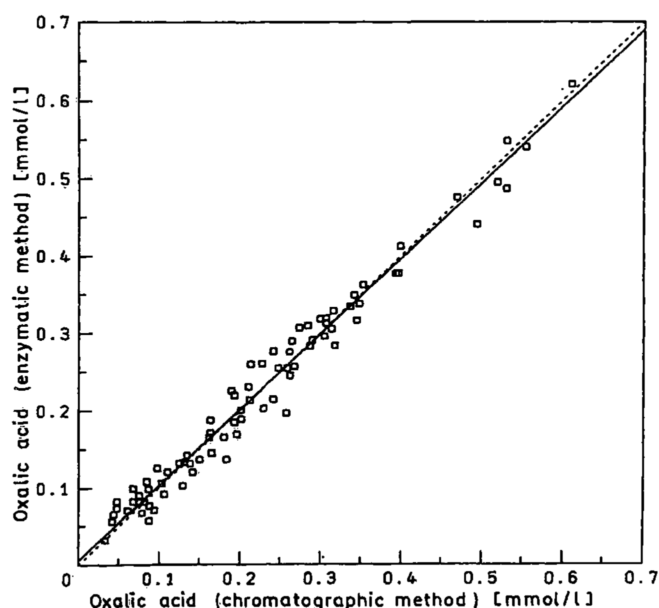

Fig. 1. Parallel determination of oxalic acid in 80 urine samples with the enzymatic and the chromatographic method: $y = 0.977x + 0.006$; $n = 80$, $y = x$ (---), regression function (——).

As *Hodgkinson* (12) showed, in order to dissolve any calcium oxalate sediment, the urine must be acidified to a pH less than 2. In the methods we tested this was achieved by adding HCl. In the enzymatic method, the use of EDTA ensures the absence of calcium oxalate sediments on subsequent adjustment to pH 5. A number of recent publications have addressed the problem of the oxidation of ascorbate to oxalate, especially in an alkaline milieu (1, 5, 8, 13, 14). With the ion chromatographic method, where the eluent has a pH of around 10, oxidation of the ascorbate is prevented by using boric acid to dilute the sample (5, 8). In the enzymatic method, where decarboxylation takes place at pH 5, high ascorbate concentrations cause interference, as ascorbate can apparently mask the NADH, which is measured photometrically. Hence, where necessary, ascorbate is removed using a simple enzymatic process. Neither method requires the prior separation of the oxalic acid from the other constituents of the sample, and hence loss of oxalate is excluded.

We believe that the enzymatic method employed here possesses certain important advantages over another commercial available enzymatic method based on oxalate oxidase (Sigma, St. Louis, USA). In the latter method, the manufacturer recommends acidifying the urine only to pH 3. Further, oxalate has to be separated from other constituents of the sample in an extra step, as oxalate oxidase is considerably more vulnerable to inhibition through various substances in the urine than is oxalate decarboxylase (15). And at least the alkaline washing stage required also means that there is a danger that any ascorbate present may be oxidized to oxalate (14).

Our results show that ion chromatographic determination of oxalic acid and the enzymatic method employed here with oxalate decarboxylase both procedure results of high and comparable realibility. They differ, however, with respect to their practicability. Taking into consideration the cost of the system, including the integrator and the recorder, the interest payments, depreciation, maintenance costs and the time required to make the analyses, the capital and material costs of the ion chromatographic method amount to about DM 5.— per determination. Assuming that a photometer is already available, the material costs of the enzymatic method amount to about DM 20.— per determination. The labour costs involved have not been calculated as the assumptions for this aspect vary greatly and are scarcely comparable between the laboratories. However, if it is assumed that one person is employed full time on the oxalic acid determination, then it would be possible to analyse approximately 25 samples per day using the ion chromatographic method, and about 40 using the enzymatic method. This means that ion chromatography is approximately 1.6 times more expensive than the enzymatic method in terms of labour costs. Considering that in the case of ion chromatography labour is in fact only required for putting the system into operation, preparing the samples and injection, it is obvious that productivity could be greatly increased by using an autosampler. This would also permit the quota of samples worked to be increased as, in this case, the system can also function outside the normal working hours. This however would increase the system price — assuming that an integrator is already available — to something in the area of 15000.— to 25000.— DM. In the case of the enzymatic method, the throughput of samples is limited by the requirement to set the pH level, although this can be facilitated by using relatively fixed volumes. A clear reduction (halving) of the material costs per analysis can be achieved by working on a semimicro scale, and not on a macro scale as we did. The volumes to be pipetted will, however, become very small in some cases, which may well affect the precision and accuracy of the results.

Thus, on the basis of our results, and considering the oxalic acid analysis only, we conclude that at a daily rate of 8—10 samples it would be sensible to employ an ion chromatograph. It remains to be seen whether the use of a recently introduced continuous-flow system (4), especially where a large number of samples (> 40) per day is being processed, will provide any alternative. Where the number of samples is small, the enzymatic method discussed here can be employed without any loss of analytical reliability.

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Priv.-Doz. Dr. A. Hesse
Experimentelle Urologie in der
Urologischen Universitätsklinik
Sigmund-Freud-Straße 25
D-5300 Bonn 1