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Ion chromatographic determination of plasma oxalate in healthy subjects, in patients with chronic renal failure and in cases of hyperoxaluric syndromes[a]

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ABSTRACT

An ion chromatographic procedure for the determination of plasma oxalate is proposed, in which the ultrafiltered sample is injected into an ion-chromatographic system. Sample processing appears effective in avoiding spontaneous oxalogenesis. Sensitivity (down to 1.0 μmol/l) allows determinations in normal and pathological samples; recoveries from plasma ultrafiltration are 94.6 ± 11.7%. Protein binding was investigated and precautions to improve recoveries from plasma ultrafiltration are proposed. The technique is simple to perform and rapid enough to be useful for routine purposes. Plasma oxalate concentrations from healthy controls averaged 6.75 ± 2.62 μmol/l (mean ± S.D. n = 18); samples from patients with primary hyperoxaluria and chronic renal failure undergoing regular dialysis were also analysed and some of the data obtained are reported and discussed.

INTRODUCTION

The tendency of calcium oxalate salts to precipitate from body fluids has been reported in some cases of end-stage renal disorders and hyperoxaluric syndromes[1]; this finding, which suggests the opportunity for a careful evaluation of calcium oxalate saturation in plasma, requires the accurate determination of plasma oxalate (OX). Most of the techniques for the direct determination of plasma OX yield higher values than the indirect *in vivo* radioisotopic dilution methods, from which mean OX

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concentrations in normal plasma are reported to range between 1 and 2 μmol/l[2–5].

Wide ranges of OX concentrations as determined by direct methods from normal plasma have been reported, which is an indication of the instability of native OX. After the earliest work, which yielded OX levels over 20 μmol/l[6,7], reported values progressively decreased and now the ranges found for normal subjects are approaching those obtained by indirect methods.

The enzymatic conversion or the chemical degradation of potential precursors on the one hand and the unfavourable ratio of interfering substances to blood OX on the other mainly account for the above overestimation[8,9]; in addition, improper sample handling may easily promote the degradation of chemical precursors, enhancing the overestimation of the native OX[10].

Enzymic methods, in which oxalate oxidase (EC 1.2.3.4) or oxalate decarboxylase (EC 4.1.1.2) are used, are available[8,11–13]. The former has been used, after plasma ultrafiltration, to convert OX to carbon dioxide and hydrogen peroxide, which can be measured spectrophotometrically by a Trinder-type reaction. The procedure, automated by using immobilized enzyme in a continuous-flow analyser, yields normal plasma OX which averages 2.03 μmol/l[11]. Oxalate decarboxylase, coupled with formate dehydrogenase (EC 1.2.1.2), has been used after precipitation of OX from plasma ultrafiltrate. With this procedure, variations of the NADH concentration, which are related to the OX content, are measured photometrically. The normal OX values averaged[8] 1.25 μmol/l.

Gas chromatographic (GC) methods have been proposed in which plasma OX, extracted by a liquid–liquid procedure, is determined as the trimethylsilyl derivative. Normal plasma OX values averaging 2.8 ± 1.1 (ref. 14) and 4.9 ± 0.8 μmol/l (ref. 15) have been reported.

However, the above techniques are tedious, time consuming and require complex manipulations of samples, so that the development of simpler and more suitable routine methods is desirable. The method reported in the present paper is a much simpler alternative; it is based on ion chromatography and allows the quantification of OX in healthy subjects and patients with chronic renal failure.

Ion chromatography is a widely used and highly sensitive technique for the determination of urinary OX[16,17]. The main problem concerning this technique is the in-column conversion of endogenous ascorbate (ASC) to OX, which can be avoided either by ASC oxidation with iron(III) ions[18] or with ascorbate oxidase[19] or by means of boric acid dilution[20]. The much higher ASC:OX concentration ratio in plasma than urine requires more careful control of the above drawback.

The chromatographic procedure allows both ASC and glyoxylate interferences to be offset both during the sample handling and in the chromatographic step. In addition, the use of specific inhibitors of the enzyme-induced oxalogenesis during plasma collection, which has been proposed previously[13], in our hand is not effective in decreasing the normal range. Samples from normal subjects and patients with chronic renal failure and primary hyperoxaluria have been analysed and some of the data obtained are reported.

EXPERIMENTAL

Reagents

Analytical reagent-grade chemicals and deionized water were used for dilutions and eluents. Sulphuric and hydrochloric acid, sodium carbonate and sodium hydrogencarbonate were purchased from Merck (Darmstadt, F.R.G.), sodium glyoxylate monohydrate from Fluka (Buchs, Switzerland), allopurinol and DL-β-phenyllactic acid from Sigma (St. Louis, MO, U.S.A.) and ascorbic acid and oxalic acid from Carlo Erba (Milan, Italy).

[^{14}C]Oxalic acid 27 mCi/mmol (Amity-Pg Amersham, Milan, Italy) was used for recovery tests. Aliquots were diluted with ethanol to obtain a concentration of 50 μCi/ml; 40 μl of solution were then diluted to 2 ml with water. A 100-μl volume of this solution was used for labelled addition to 1 ml of plasma. PicoFluor (Packard Instruments, Zurich, Switzerland) was used, as a scintillation solution, at a dilution ratio of 60:1 of sample. Radioactivity was measured using a Model 81000 liquid scintillation counter (LKB, Cambridge, U.K.).

A combined inhibitor solution containing allopurinol and boric and DL-β-phenyllactic acids was prepared and used as described elsewhere[13]. The OX stock standard solution was prepared by dissolving 504 mg of oxalic acid dihydrate in 10.0 ml of water and stored at −20°C until used. Working standards of 10 and 100 μmol/l were prepared daily by diluting the above concentrated solution.

Sample handling

Human blood from fasting healthy subjects was obtained by venepuncture using heparinized Vacutainer vessels. Blood from haemodialysed patients was withdrawn by arterio-venous fistulas before and after dialysis. The collections, immediately placed in melting ice, were centrifuged at approximately 1000 *g* for 10 min at 4°C as soon as possible. A 1.0-ml volume of separated plasma was acidified by addition of 40 μl of concentrated hydrochloric acid. The sample was vortex mixed vigorously for 6 min and was transferred to an MPS-1 ultrafiltration unit (Grace Italiana Divisione Amicon, Passirana di Rho, Milan, Italy); ultrafiltration was performed by centrifugation at 4°C at 1500 *g* for 15 min using YMT membranes (Amicon) with a molecular weight cut-off of 30 000 daltons; ultrafiltration allowed the separation of about 100 μl of liquid, which was harversted and diluted five-fold with a 0.3 mol/l boric acid solution and then injected into the chromatograph.

The efficiency of ultrafiltration was tested by radioisotopic dilution. To the separated plasma samples were added 100 μl/ml of the above 1 μCi/ml solution of [^{14}C]oxalic acid and then treated as described. A 50-μl aliquot of sample was mixed with 3.0 ml of scintillation liquid and the emulsion was left overnight at 4°C and then measured for radioactivity. The recovery of [^{14}C]OX from the ultrafiltration step was evaluated by comparing the measured radioactivity of the treated samples with that of the non-ultrafiltered samples.

Chromatography

The chromatographic separation was performed with a QIC liquid chromatograph (Dionex, Sunnyvale, CA, U.S.A.) equipped with a conductimetric detector. Two in-line AS-4A anion separator columns (Dionex, 037041) in conjunction with an

AG-4A guard column (Dionex, 037042) were used as a stationary phase. An automatic injection valve connected with a 200-μl sample loop was used. A sodium carbonate–hydrogen carbonate (2.4 and 3.0 mmol/l) aqueous solution pumped in the column at 2.0 ml/min was used as mobile phase, after accurate degassing.

Periodic replacement of the precolumn and clean-up of separators are essential for the best chromatographic performance. Regeneration was carried out by flushing the separators with 100 ml of 0.2 mol/l sodium hydroxide solution. Columns were ready for analysis after 1 h of conditioning. Eluent conductivity background was suppressed with a cation-exchange membrane (Dionex, 038019) set in-line after the separator. The membrane, in which the eluent flows, is plunged in a counterflow batch of 12.5 mmol/l sulphuric acid flowing at 2.7 ml/min. In the membrane suppressor, eluent and sample anions do not permeate the cation-exchange membrane because of electrostatic exclusion forces, but cations do. Owing to the selective exchange between sodium and hydrogen ions, sodium carbonate and sodium hydrogencarbonate are converted to weakly conducting aqueous carbon dioxide, whereas sodium oxalate is converted to highly conducting oxalic acid. The signal-to-noise ratio is consequently improved. The detector output was set at 100 μS. Oxalate peak heights were measured using a Spectra-Physics SP 4270 plotter/integrator set at an attenuation input ranging from 4 to 32 mV full-scale.

RESULTS

Removal of proteins and macromolecules from the sample was accomplished by plasma ultrafiltration. In addition, some tests were performed in order to investigate and improve the conditions of ultrafiltration with respect to the recovery of OX and the stability of the analyte. The recovery of OX, as tested by isotope dilution, was evaluated as a function of plasma acidification. In particular, plasma samples containing [^{14}C]OX were spiked with various amounts of concentrated hydrochloric acid, vortex mixed for 6 min and subsequently ultrafiltered. The recoveries were substantially quantitative for unacidified plasma and decreased as acidification increased towards a minimum value averaging 56.7%, corresponding to an approximate plasma pH of 3. Further acidification allowed an increase in recovery, reaching up to 94.6% at pH < 1; in addition, a notable increase in precision was observed (Table I).

TABLE I

[^{14}C]OX RECOVERY MEASURED AFTER ULTRAFILTRATION OF PLASMA SAMPLES TREATED AS DESCRIBED IN THE TEXT

Parameter	*Unacidified*	*HCl-acidified (μl HCl/ml plasma)*			
		10	*20*	*30*	*40*
No. of samples	8	9	16	22	161
Mean recovery (%)	105.3	56.7	67.4	71.0	94.6
S.D. (%)	8.4	22.9	16.1	13.2	11.7
Relative S.D. (%)	8.0	40.4	23.9	18.6	12.4

TABLE II

SPONTANEOUS *IN VITRO* OXALOGENESIS IN DELAYED ANALYSIS OF ULTRAFILTRATES FROM THE SAME PLASMA SAMPLE, BOTH ACIDIFIED AND UNACIDIFIED, SPIKED WITH GLYOXYLATE AND ASCORBATE

Delay time (h)	*Oxalate concentration (μmol/l)*				
	Native plasma		*Spiked with 100 μmol/l of glyoxylate, acidified*	*Spiked with 100 μmol/l of ascorbate*	
	Acidified	*Unacidified*		*Acidified*	*Unacidified*
0	6.4	8.5	8.0	–	–
1	7.0	12.2	9.3	7.0	21.4
3	7.7	19.4	–	9.6	32.0

These findings suggest that OX is entirely unbound at physiological pH values. Mild acidification (10 μl of HCl per ml of plasma) enhances the above binding, which can be broken in turn by further acidification ($\geqslant 30$ μl of HCl per ml of plasma). This evidence is in agreement with the expected trend of the reciprocal electrostatic affinity occurring as the degree of protonation of both OX and proteins increases. As more satisfactory conditions were obtained from both the untreated plasma and the plasma acidified with 40 μl of HCl/ml, further studies were performed using the above procedures. The ultrafiltration of untreated plasma yielded a large volume of ultrafiltrate, the analysis of which produced well resolved chromatograms, but the OX generation during the preanalysis delay was not negligible. In addition, spikes of ASC sharply increased both the basal OX and the oxalogenesis rate (Table II).

The acidification of plasma inhibited the spontaneous oxidation of ASC (Table II) and suppressed the enzyme-induced production of OX by the immediate denaturation of proteins. Concentrations from normal subjects averaged 6.75 μmol/l, and were thus in fairly good agreement with the results of previous GC[15] and high-performance liquid chromatographic methods[21]. The acidification procedure, even though it suppresses the oxalogenesis at native pH, provides chromatographic traces of worse quality and lower ultrafiltration rates.

Tests were carried out by ultrafiltering untreated plasma and by collecting the ultrafiltrate directly in HCl. Its low concentration (7 μl of 2 mol/l HCl in about 150 μl of final ultrafiltered sample) on the one hand inhibits oxalogenesis and on the other does not reduce the quality of the chromatograms. Nevertheless, the resulting OX concentrations as measured in normal subjects were slightly higher than those obtained by ultrafiltering acidified plasma, indicating that a certain oxalogenesis occurs in the course of the ultrafiltration at physiological pH. Therefore, immediate acidification of plasma with 40 μl of HCl per ml was adopted.

A number of samples were analysed by collecting blood in the presence of a mixture of inhibitors, as suggested in the literature[13], in order to decrease the hypothetical rapid conversion of glyoxylate or glycolate into OX during the sample harvesting. No improvements were observed and this procedure was discarded.

Calibration and sensitivity

The analysis of both untreated aqueous solutions containing oxalic acid in the range 0–100 μmol/l and of treated samples spiked with known amounts (0–100 μmol/l) of oxalic acid produced satisfactory linear responses.

While the chromatographic traces from standards and unacidified plasma permit a minimum detectable concentration of 0.5 μmol/l, the sensitivity of analysis for acidified plasma is limited by a disturbed signal in which OX elutes on a tailing peak. This drawback, possibly due to the fragmentation of metabolites enhanced by acidification with likely production of dicarboxylic acids, could not be avoided by any modification of the chromatographic conditions. However, the minimum detectable concentration was 1.0 μmol/l, which is much lower than the normal range found here. Otherwise, samples from uraemic patients could be easily analysed because of the higher OX content.

Fig. 1 shows three chromatograms, representing analyses of a standard, of plasma from a healthy person and of plasma from a patient on dialysis treatment.

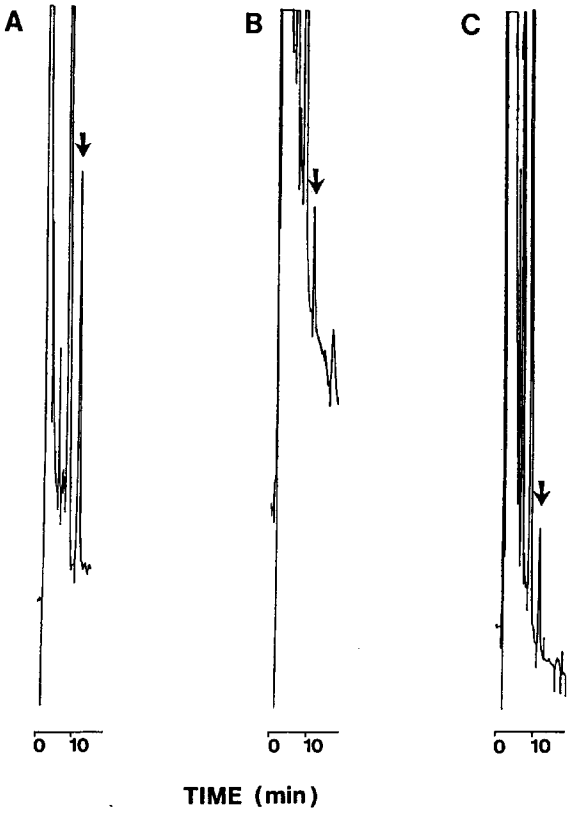

Fig. 1. Typical ion chromatograms for the determination of oxalate under the conditions described in the text. (a) Oxalate standard, 20 μmol/l in water, 4 mV full-scale; (b) plasma sample from a healthy person, 4 mV full-scale; (c) plasma sample from a patient on regular haemodialysis, 16 mV full-scale. Detector sensitivity was set at 100 μS. The arrows show oxalate peaks.

Accuracy and precision

Four ultrafiltrates were analysed both immediately and after storage for 24 h at −20°C. The OX content changed from 12.0 ± 6.9 to 27.1 ± 10.4 μmol/l (mean ± S.D., $p = 0.03$). Even though normal plasma glyoxylate levels would not exceed 0.3 μmol/l, as observed using a previously published method[22], or 2.5 μmol/l as reported by other workers[23], the possible interfering effects of ASC and glyoxylate were tested by spiking plasma with 100 μmol/l of each. The results showed that the sample processing was efficient in avoiding any appreciable overestimation of the measured OX (Table II). More generally, under the proposed conditions, oxalogenesis has to be considered insignificant, provided that the injection is carried out within 2 h (Table II).

As no increase in measured OX was observed when 200 μmol/l of ASC was added just before injection, no significant overestimation can be ascribed to the in-column conversion of native ASC.

Two plasma samples, from a healthy subject and ureamic patient, were analysed four times within the same series. The samples contained OX concentrations averaging 6.8 and 38.8 μmol/l and the relative standard deviation averaged 11.8 and 4.5%, respectively.

The residual tendency for oxalogenesis does not allow the treated samples to be stored and an inter-run precision to be measured. A raw estimate of the between-run precision was obtained by analysing two pre-dialysis samples from fifteen patients withdrawn at subsequent dialysis sessions (range 26.1–75.1 μmol/l). The result, expressed as standard deviation, S.D. $= [(\Sigma d^2)/2n]^{\frac{1}{2}}$, was 3.53 μmol/l.

The ultrafiltration recovery was evaluated in plasma by radioisotopic dilution (Table I). The recovery from the chromatographic step was investigated. The addition of 40 μmol/l of OX to six given ultrafiltrates yielded a mean recovery of 100.4 ± 8.6%.

Clinical results

Samples from 18 apparently healthy adults and 20 patients with chronic renal failure undergoing regular haemodialysis (3–4 h, three times a week) were analysed. The normal OX concentration ranged from 1.42 to 10.70 μmol/l (mean ± S.D. = 6.75 ± 2.62, $n = 18$). Pre-dialysis values averaged 48.1 ± 15.9 μmol/l (mean ± S.D., $n = 20$) and were significantly higher than post-dialysis values ($p < 0.001$) which averaged 18.0 ± 10.1 μmol/l (mean ± S.D., $n = 20$). Samples from three patients with primary hyperoxaluria, two of whom were undergoing regular dialysis treatment for end-stage renal failure, were also investigated. Pre and post-dialysis values for the latter-two were 157.0 and 40.5 μmol/l and 197.6 and 81.4 μmol/l. The third patient, whose renal function was only mildly reduced, had a plasma OX level of 21.4 μmol/l.

CONCLUSIONS

The proposed method is based on the purification of OX by ultrafiltration and subsequent ion-chromatographic assay of the ultrafiltrate. In our hands, ultrafiltration was found to be an efficient procedure for separating the main fraction of proteins, and thus for decreasing the enzyme-induced OX formation. The trend of ultrafilterability as a function of the plasma pH (Table I) suggests, in agreement with previous reports[8], that OX–protein binding cannot be neglected if plasma is moderately acidified. However, further acidification (pH < 1) was found to be

effective in breaking the above binding, allowing quantitative and close recoveries to be obtained.

The immediate acidification allows, first, the protein-containing fractions to be denaturated, making the time required to complete the ultrafiltration less critical, and second, the possible oxalogenesis due to the main interfering compound (*i.e.*, ASC) during the ultrafiltration step to be inhibited.

Results obtained by collecting blood in the presence of specific inhibitors for the enzymic oxidation of glycolate and glyoxylate into OX[13] do not differ significantly from those obtained in the absence of the above inhibition.

In spite of these precautions, the normal range obtained is higher than that reported by using indirect methods[2–5]. The direct methods proposed so far do not provide homogeneous data, which are generally higher than those obtained by *in vivo* isotopic dilution. A few workers have obtained mean values ranging from 1 to 3 μmol/l (refs. 8, 11 and 14) using enzymatic or GC techniques.

On the basis of these considerations, our overestimation could be ascribed to the chromatographic step. Unfortunately, ultrafiltrates incubated with commercially available suspensions of oxalate decarboxylase were unsuitable for chromatographic analysis because of the high saline and the low OX contents. Nevertheless, the above eventuality seems unlikely, as the only known chromatographic interference would be due to ASC, which under the described conditions seems negligible. Moreover, other workers have reported normal ranges for plasma OX to be very close to the values found here[15,21,24]. In any case, no problem seems to exist when the present method is applied to the study of patients with chronic renal failure.

As an example of the clinical usefulness of the determination of OX in plasma, samples were analysed before and after dialysis, and the resulting data were comparable to those obtained by other workers[14,25,26]. The observation that, in our hands, the recoveries of [^{14}C]OX were fairly constant suggests that this step may be considered unnecessary.

Hence, the present method, which requires a minimum volume of blood and is rapid and simple to perform, can be considered as an alternative to the methods proposed previously. Also, it appears to be sensitive, accurate and suitable for routine use, provided that one bears in mind that the analysis cannot be delayed after the blood has been collected.

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