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Liquid chromatography–tandem mass spectrometry method for routine measurement of oxalic acid in human plasma

Katja Benedikte Presto Elgstoen*

Department of Medical Biochemistry, Rikshospitalet Medical Centre, 0027 Oslo, Norway

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ABSTRACT

A solid phase extraction (SPE)-LC–MSMS method for the routine determination of oxalic acid (OX) in plasma, a diagnostic marker of primary hyperoxaluria (PH), was developed and validated. The normal range of OX was found to be 3–11 μmol/L ($n = 67$), with no differences attributable to gender or age. The effect of pre-analytical factors on the *in vitro* production of OX was investigated, and plasma was found to be stable for 1–2 h at room temperature, less after ingestion of vitamin C; the process was not completely stopped by preservation at either −20 or −70 °C.

1. Introduction

OX is a diagnostic marker for two rare inherited metabolic diseases: primary hyperoxaluria type 1 (PH1); and the usually milder primary hyperoxaluria type 2 (PH2) [1]. PH1 is caused by a deficiency of the liver-specific peroxisomal enzyme alanine:glyoxylate transaminase (EC 2.6.1.44), while PH2 is caused by a deficiency in the cytosolic enzyme glyoxylate reductase/hydroxypyruvate reductase (EC 1.1.1.79). Both enzyme deficiencies result in increased synthesis of OX. In addition, an increased level of glycolic acid is found in PH1, and L-glyceric acid in PH2 [1]. OX is eliminated by renal excretion, but because of poor solubility of calcium oxalate, it forms deposits in the kidneys. Renal failure typically develops later, followed by calcium oxalate deposition in bone, blood vessels, myocardium, and other organs [2]. However, as patients approach end-stage renal failure, the urine excretion of OX normalizes and the concentration of the metabolite in blood increases.

OX can also be secondarily elevated in several other clinical conditions such as chronic renal failure, urinary stone disease and intestinal malabsorption. Measurement of the OX concentration in biological fluids may therefore provide important diagnostic information in various medical conditions. As the laboratory findings in medical conditions like PH which affect renal function differ during the course of the disease, there is a need for both plasma and urine analysis of OX.

For measurement of OX in urine, but not in plasma, commercial kits are available. During the last seven decades, a number of different methods for plasma determination of OX have been reported, but none of them has reached widespread use, and the proposed normal concentration range of OX varies extensively (see Table 1). The discrepancy between the normal ranges published can partly be ascribed to variable specificity between methods used, e.g. the underestimation of OX due to binding of OX to proteins in analytical procedures involving protein precipitation at low pH [3].

We therefore found the need for the development of a state-of-the-art, fast, and reliable method for quantitation of OX in plasma.

For the isolation of organic acids from body fluids, the use of SPE with SAX material has been reported [4].

Being a strong acid (pK_{a1} 1.23 and pK_{a2} 3.83), OX was therefore expected to be suitable for SPE SAX-extraction. In addition, SPE-procedures can easily be automated using robots. Automation reduces the need for labour-intensive manual procedures and makes automated SPE attractive in routine laboratories. For more than 40 years, liquid–liquid extraction followed by GC–MS analysis has been used in our laboratory for routine analysis of organic acid in urine. However, the GC–MS analysis time is more than 40 min and the recovery of oxalate from the liquid–liquid extraction is poor. LC–MSMS operated in MRM-mode has become one of the most powerful analytical techniques for routine

* Tel.: +47 23073079; fax: +47 23070902.
E-mail address: katja.elgstoen@rikshospitalet.no.

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Table 1
Reported normal ranges of OX in plasma using different analytical methods

Method	Normal range (μmol/L)	*n*	Reference
Titrimetric, cerium salt	26–85	9	[19]
Fluorimetric, calcium salt	15–31	15	[20]
Radioisotopic, *in vivo*	0.75–2.11	7	[21]
Radioenzymatic	8.6–15.7	6	[22]
Fluorimetric	13–27.8	20	[23]
Enzymatic, oxalate decarboxylase	0–5.4	11	[5]
	<0.8–1.5	35	[24]
	8–22[m]	24	[9]
	15–51[f]	16	[9]
Radioisotopic	0.1–0.9	3	[25]
Enzymatic, oxalate oxidase	1.3–3.1	21	[26]
	2.03[m b]	12	[26]
	2.25[f b]	9	[26]
	0.4–3.7[d]	49	[15]
	<10–55[a]	28	[27]
	3.0–7.5	73	[28]
	1.4–6.0[a]	30	[12]
	(0–3y):1.1–4.3[a]	10	[12]
	(8–14y):1.4–6.0[a]	30	[12]
	(0.1–17y):0.78–3.02	33	[14]
LC-enzymatic, oxalate oxidase	<0.68–15.9[a]	133	[13]
LC-electrochemical	11–27	4	[29]
LC-conductivity	1.4–2.5[m a]	11	[30]
	0.7–2.9[f a]	12	[30]
	1.42–10.7[a]	18	[10]
	0.8–3.4[a]	31	[31]
	1.7–2.2[a c]	39	[32]
	1.18–2.49[m]	10	[33]
	1.31–2.5[f]	6	[33]
	(0.2–17y):5.37–7.49	50	[34]
GC-flame ionization	8.9–41	40	[35]
	1.3–5.3	22	[7]
GC–MS	1.7–3.9[a]	8	[36]
Current procedure, LC–MSMS	2.5–13	67	

y, age in years; m, male; f, female; *a*, fasting; *b*, mean value (standard deviation); *c*, no vitamin C supplement in previous week; *d*, oxalate restricted diet for 2 days.

quantitation of diagnostic metabolites in body fluids. The combination of both high selectivity and sensitivity probably explains the increasing popularity of this hyphenated technique in clinical laboratories, and makes it a natural choice for development of new analytical methods.

In addition to the variable specificity between the earlier reported methods, the *in vitro* conversion of blood constituents to OX during storage, after collection ("*in vitro* oxalogenesis"), most certainly plays a critical role in the measurement of OX in plasma. The phenomenon has been known for many decades, and potential precursors of OX, and suggested procedures to prevent the *in vitro* oxalogenesis have been described by many investigators. In 1980, Akcay et al. [5] proposed that an enzymatic conversion of glyoxylate to oxalate was the major source of the *in vitro* OX production. Several investigators [6,7] tried, but failed, to reproduce these findings.

In contrast, the breakdown of vitamin C to OX [8] has been generally accepted as a major source of *in vitro* oxalogenesis. Sample preparation procedures involving adjustment of the pH of plasma or serum shortly after sample collection have been reported by several investigators. Hatch et al. [9] adjusted the pH of serum to 10.6 by adding KOH, while other investigators have claimed that stabilizing vitamin C by prompt acidification [7] followed by ultracentrifugation [10] of plasma is critical to avoid *in vitro* oxalogenesis. A special assay-like plasma OX is likely to be implemented only at relatively large medical centers. Thus one can expect samples to be sent from laboratories lacking specialized equipment, and assayed up to several days later. For this reason knowledge of the effect of pre-analytical factors on the *in vitro* oxalogenesis is of great importance.

The aim of the reported work was to develop a fast and reliable SPE-LC–MSMS method for routine determination of OX in plasma to be implemented as a diagnostic tool for PH, to evaluate how pre-analytical factors influence the results, and to find the normal range of OX in plasma. The influence of vitamin C, a known precursor of OX, and the addition of potential inhibitors of the *in vitro* oxalogenesis process were also tested.

2. Experimental

2.1. Chemicals and materials

All chemicals used were of analytical grade. OX was purchased from Sigma (St. Louis, MA), and vitamin C (ascorbic acid) from Merck (Whitehouse Station, NJ). The isotopically labeled internal standard, oxalic-1,2-$^{13}C_2$ acid ($^{13}C_2$OX), was obtained from Cambridge Isotope Laboratories (Andover, MA), and radioactive-labeled oxalic-1,2-$^{14}C_2$ acid ($^{14}C_2$OX) (1 mCi with specific activity 5 mCi/mmol) from American Radio Chemicals Inc (St. Louis, MO). Acetyl chloride was from Fluka (Buchs, Switzerland), and methanol and acetonitrile of HPLC grade from Rathburn (Walkerburn, Scotland). Water used for both sample preparation, dilution of standards and preparation of solvents for chromatography and SPE was deionized and purified (to a total resistance of 18 MΩ and a total content below 10 ppb) using a Millipore Synthesis water purification system (Millipore GmbH, Germany).

Stock solutions of 25, 50, 125, and 250 μmol/L OX, and 125 μmol/L of $^{13}C_2$OX were prepared in water and stored at −20 °C. All solvents for LC–MSMS and SPE were freshly made every week. The derivatization reagent, 10% acetyl chloride in butanol, was stable for several months when stored in refrigerator at +4 °C.

For absolute recovery experiments, $^{14}C_2$OX was dissolved in water, and an amount giving a total count of 50,000 dps (10 μmol/L OX) was used. Emulsifier-Safe purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA) was used as liquid scintillation counting-cocktail. For SPE, strong anion exchange columns (Varian SAX, 100 mg/1 mL) were used. The chromatographic separation was performed at ambient temperature on a Betasil C8 Pioneer column (50 mm × 21 mm, 5 μm particles) purchased from Thermo (Waltham, MA).

2.2. Instrumentation

The β-emission of the radio-labeled OX was counted in a Tri-Carb 2300TR Liquid Scintillation Analyzer from Packard. SPE was performed on a Gilson ASPEC XL4 robot.

For the LC–MSMS separation, a PerkinElmer series 200 HPLC system with two pumps and an auto-injector was interfaced with an API 2000™ triple quadrupole MSMS (Applied Biosystems/MDS Sciex) equipped with an electrospray source. The built-in diverter valve on the MSMS was used to switch between waste and MSMS.

2.3. Standard and plasma sample preparation

Blood was drawn into heparin-containing tubes and without delay centrifuged at 1740 g for 10 min at room temperature. Unless processed immediately, plasma was stored at −70 °C until analysis.

All calibrators and plasma samples were processed and analyzed in duplicate.

Calibrators at 0 (blank), 5, 10, 25 and 50 μmol/L OX respectively were prepared for the calibration curve, and a 7.5 μmol/L OX solution included as quality control. For plasma samples, 500 μL was used. To all samples, internal standard was added to a final concentration of 25 μmol/L $^{13}C_2$OX. In addition, one double blank,

containing water only, was included in each analytical series. All samples were wortexed well and placed on the SPE robot.

For SPE, the SAX-columns were first equilibrated with acetonitrile, followed by 5% acetonitrile in water. After loading of the sample, the cartridges were cleaned using the same solvents, but in reversed order. Finally, 600 μL of 0.4 mol/L hydrochloric acid in acetonitrile was used to elute OX from the SAX cartridge.

The vials were then evaporated to dryness in a 30 °C water bath using a gentle flow of nitrogen. After addition of 100 μL of 10% acetyl chloride in butanol, the derivatization was completed in 15 min at 65 °C. Excess reagent was removed by evaporation as described above, but with lower gas flow. The sample was then redissolved in 50% ACN in water.

2.4. LC–MSMS procedure

OX and $^{13}C_2$OX were separated from background ions on a C8-column using a mobile phase of 60% acetonitrile in water.

After elution of OX and $^{13}C_2$OX at about 3 min, a linear gradient from 60 to 100% acetonitrile for 1 min was used. The system was kept at 100% acetonitrile for 5 min to remove strongly retained compounds from the LC-column. A linear gradient for 1 min back to the initial conditions, followed by equilibration for 2 min, was performed before the next injection at 13 min. The diverter valve was switched between waste and MSMS to minimize the contamination of the MSMS.

The MRM-transitions of 203–57 for OX and 205–57 for $^{13}C_2$OX were used for MSMS-quantitation. The electrospray voltage was set at 5500 V and the collision energy 15 V. The curtain gas was at 40 psi, nebulizer gas at 60 psi, and a heating gas at 70 psi, delivering nitrogen at 400 °C, was used.

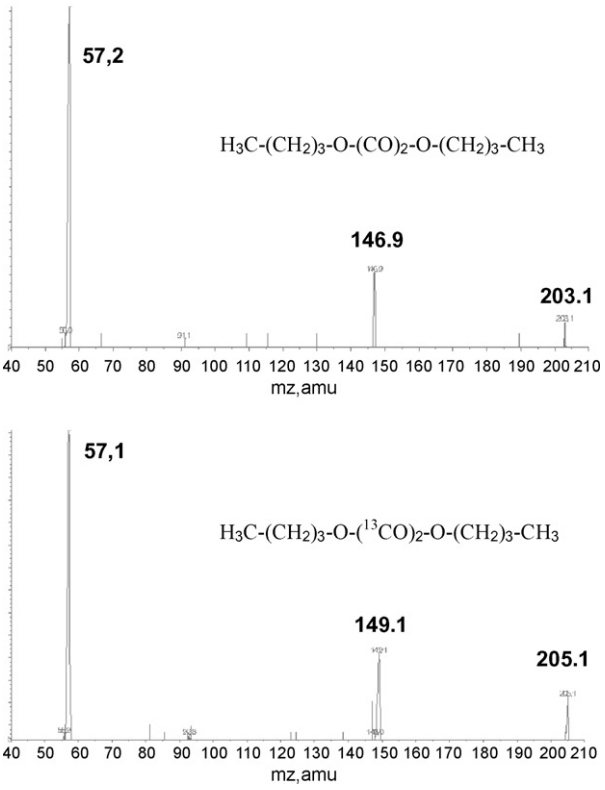

Fig. 1. Tandem mass spectrometry fragmentation pattern of OX (upper panel), and $^{13}C_2$OX.

3. Results and discussion

3.1. SPE-LC–MSMS method development

For the SPE-procedure, as ion exchange kinetics is somewhat slower than other retaining mechanisms, the robot was programmed to use low dispensing flow and to work in a sequential mode; that is, all steps were completed for all cartridges until the next step in the extraction procedure.

To tune and optimize the mass spectrometer for OX and $^{13}C_2$OX, standard solutions were infused into the MSMS. OX produced a molecular ion in negative mode at m/z 89, but the abundance of this ion was too low to be used for quantitation of the acid in plasma. Recently, Keevil and Thornton [11] demonstrated urine analysis of OX using MS in negative mode, but the concentration of OX in urine is considerably higher than in plasma. To improve the ionization efficiency of OX before the LC–MSMS analysis, we converted the acid to different corresponding alkyl-esters, namely dipropyl, dibutyl and dipentyl esters. All alkyl esters tested gave satisfactory MRM-transitions, but the dipropyl esters were shown to be too volatile, resulting in unacceptable sample loss; and the evaporation and chromatic-separation times required by the dipentyl esters were considered unacceptably long. As a result, the method development was continued using the dibutyl esters. The protonated molecular ion $[M+H]^+$ for the analyte, and the internal standard, were found at m/z 203.1 and 205.1, respectively. The most abundant fragment ion for both OX and $^{13}C_2$OX was found at m/z 57 (Fig. 1). The transitions of (205.1–57.1) for $^{13}C_2$OX and (203.1–57.1) for OX were therefore chosen as MRM-transitions.

Good chromatographic performance was easily obtained using a mobile phase of 60% acetonitrile in water, and the early elution of OX on the C8-column made it possible to inject a new sample after only 4 min. However, when analyzing large batches of plasma samples, contamination of both the ion source and the chromatographic column was sometimes observed. A cleaning step, using a gradient from 60 to 100% acetonitrile was therefore included to remove strongly retained impurities.

Although the lifetime of the column was greatly prolonged by including the cleaning step, contamination of the ion source was at the same time markedly increased. Therefore, the diverter valve on the MSMS was set to waste for the first 2 min, when unretained impurities eluted, and during the cleaning step. MRM-data was collected only between 2 and 4 min after injection, during the time when OX eluted from the column. In this way, contamination of the MSMS was minimized and unnecessary MSMS data collection avoided.

A summary of the time programme and a LC–MSMS trace from analysis of a plasma sample is shown in Fig. 2.

3.2. Assay validation

The assay was found to be linear from 1 to 200 μmol/L OX when analyzing aqueous standards. The limit of quantitation (LOQ) for OX in plasma was 0.5 μmol/L. Within-day repeatability (all preparative and analytical processes included) was tested by assaying 10 replicates of both a 7.5 μmol/L aqueous standard, and plasma containing three different levels of oxalic acid (4, 12, and 50 μmol/L OX). A CV of 4.5% was found for the aqueous standard, and an average CV of 6.9% for the plasma samples.

The LC–MSMS repeatability, calculated by injecting the same sample 10 times, was found to be 1.7% for a 7.5 μmol/L aqueous standard and 3.0% for a plasma pool containing 12 μmol/L OX.

Because of *in vitro* oxalogenesis from plasma constituents it was difficult to implement long-term plasma quality controls in the

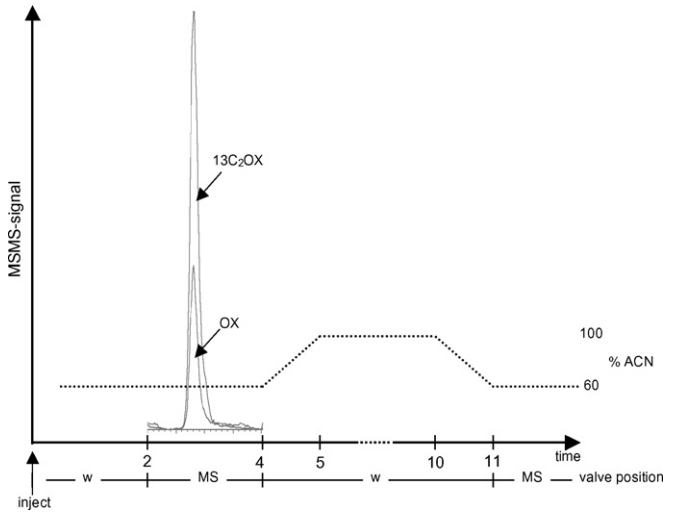

Fig. 2. LC–MSMS time programme and MS–MS trace of a plasma sample. After elution of OX and $^{13}C_2$OX at about 3 min, a linear gradient from 60 to 100% acetonitrile (dotted line) for 1 min was used. The system was kept at 100% acetonitrile in 5 min to remove strongly retained compounds from the LC-column. A linear gradient for 1 min back to the initial conditions was performed, followed by a 2-min equilibration, before the next injection at 13 min. The diverter valve was switched between waste (W) and MSMS to minimize the contamination of the MSMS.

assay. As an alternative to real plasma, we tested the use of artificial plasma [12] with added OX. Unfortunately, this resulted in clogging of the SPE cartridges and poor chromatography. Instead, a 7.5 μmol/L aqueous OX solution was implemented as quality control in each analytical series. The long-term reproducibility for the quality control was found to be 5% over a 3 months period (n = 18).

3.3. Absolute and relative recovery of OX

To evaluate the recovery of the assay, both relative recovery using $^{12}C/^{13}C_2$OX-ratio, and absolute recovery using $^{14}C_2$OX were examined.

The relative recovery was tested by spiking a plasma sample with 5, 15, 30, 50, 100 and 200 μmol/L OX. For the samples spiked with 5–100 μmol/L OX, 96–97% relative recovery was found, dropping to 93% recovery for the sample spiked with 200 μmol/L OX (data not shown). The high relative recovery observed was expected, as the physical properties of the internal standard and the analyte were very similar, thus the losses during evaporation, extraction, and ionization were expected to be nearly identical.

Absolute recovery was measured by adding a radioactive isotope of OX, $^{14}C_2$OX, to plasma from two different persons. One portion of plasma was processed immediately, while one portion was left at +4 °C for 24 h to equilibrate. At each step in the sample preparation procedure, radioactivity in an aliquot was counted and the losses of analyte during the different steps were calculated. Reduced gas flow was used for the second evaporation step, after derivatization, as the dibutyl ester of OX was expected to be more volatile than OX itself.

No significant difference in analyte loss between the fresh and equilibrated samples was found, nor between the different plasma samples (results not shown). The average absolute recovery was 93% after SPE, 82% after the first evaporation step and 25% after the second evaporation step (data not shown). These results show that the removal of excess derivatizing reagent by evaporation must be performed under very mild conditions to avoid unacceptable sample loss. Although an absolute recovery of 25% seems very low, the relative recovery, which is often referred to as "recovery", is above 90%.

To our knowledge, measurement of absolute recoveries by the use of radio-labeled isotopes is today not commonly used as part of a validation procedure. Nevertheless, most valuable information about sample loss during the different steps of a sample preparation procedure can be found with this method.

3.4. Normal range

The normal range of OX in plasma was found by analyzing plasma collected from 67 healthy volunteers, 37 females and 30 males, aged 23–71 years. The plasma OX concentrations found were not normally distributed. An approximately linear z-score plot was obtained by a log transformation of the data. No age difference was found, and the values for men and women were not statistically significantly different. The reference interval was then estimated parametrically for the log transformed data by regression analysis to z-scores, and was found to be 3–11 μmol/L, which also was confirmed by calculation of a non-parametric method.

From 1943 to today, more than 35 reports giving values of normal range of OX in plasma have been published. The number of samples analyzed in most reports is limited. Table 1 summarizes some of the analytical methods and corresponding normal ranges reported. As can be seen, the normal range found using the current procedure, 3–11 μmol/L, is in the middle range of the reported reference intervals. One report describes age-related differences in plasma OX in the adult population [13], analyzing samples from 130 healthy individuals. It was suggested that the higher OX level found in those over 60 could be caused by a decrease in renal function with age. Although we included 67 plasma samples from healthy subjects up to the age of 71 years, we were not able to confirm their findings, but we cannot exclude the possibility that we would have detected an age effect if a larger population had been studied.

In agreement with what most others have found, we observed no significant gender difference.

Very few reports on the plasma-OX concentration in children are available. Barratt et al. [14] and Porowski and Galasinski [12], found, by analyzing samples from 33 to 70 children, respectively, no significant difference in plasma-OX levels between children and adults. Due to limited availability of plasma from healthy children, we did not have sufficient data available to calculate the normal range of OX in plasma from young individuals.

3.5. Plasma OX in PH patients

Plasma from three PH patients was collected, and the concentration of OX found was in the range of 50–170 μmol/L. Samples collected before and after dialysis on 10 different days from a PH1 patient who had undergone both liver and kidney TX were also analyzed. The concentration of OX in the patient's blood was found to be very stable at 30–35 μmol/L before dialysis dropping to 15–18 μmol/L after dialysis.

3.6. Evaluation of oxalogenesis in frozen samples

To examine the effect of storage time, temperature and initial OX concentration on oxalogenesis, aliquots of plasma from each of 34 volunteers (initial OX concentration interval 2.7–13.5 μmol/L) were stored at −20 and −70 °C, respectively and analyzed after 7, 16, 30, 41, 49 and 56 days. A linear backward multiple regression model was used, and the absolute increase in oxalate concentration (Δoxalate vs. fresh sample) was chosen as the dependent variable, and storage time and initial concentration as independent variables, i.e. $\Delta\text{oxalate} = a + b \times \text{oxalate}_{\text{initial}} + c \times \text{time} + \varepsilon$, where ε is the residual. The two temperatures, −20 and −70 °C were treated separately.

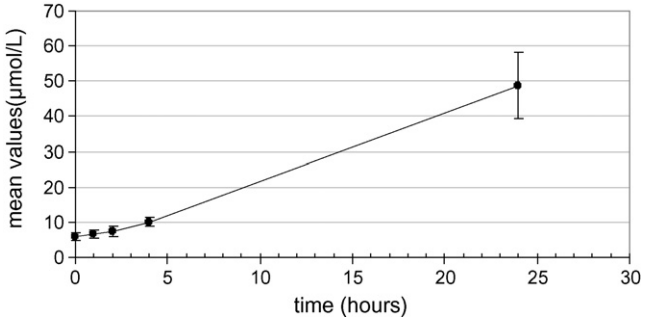

Fig. 3. *In vitro* oxalogenesis after 0, 1, 2, 4 and 24 h storage at room temperature in plasma samples from seven healthy controls. The points (●) represent the mean plasma oxalate values. (⌶) indicate SEM values.

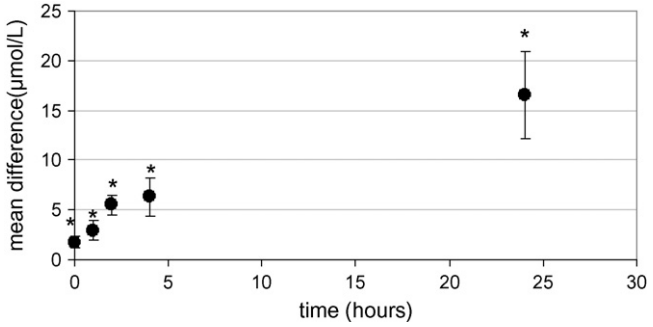

Fig. 4. Increased *in vitro* oxalogenesis after ingestion of vitamin C 2 h prior to sample collection. The points (●) represent the mean difference of plasma oxalate from five individuals between a 1-g vitamin C supplement and a normal diet. Time points represent 0, 1, 2, 4 and 24 h storage at room temperature. (⌶) indicate SEM values.

For −20 °C, we obtained $a = 6.7$ μmol/L ($P = 0.002$); $b = -0.84$ ($P = 0.0002$); $c = 0.09$ ($P = 0.0005$); $R^2 = 0.56$ ($n = 32$). For −70 °C we obtained $a = 7.4$ μmol/L ($P < 0.0001$); $b = -0.97$ ($P < 0.0001$); $R^2 = 0.54$ ($n = 34$). The first equation shows that there is a mean increase of 0.09 μmol/L in oxalate concentration per day due to storage time up to 8 weeks at −20 °C, and that the absolute increase in oxalate level is negatively related to its initial value. With preservation at −70 °C, we found no significant increase in the oxalate level with time, but a negative relationship between absolute increase and initial concentration.

There are relatively few reports on the stability of OX in frozen samples. To our knowledge, the correlation between initial concentration, storage time and temperature has not been reported previously. Maguire et al. [6] found that serum was stable up to 48 h when preserved at −20 °C, but observed a dramatic increase when vitamin C was added. Berckmans et al. [15] found that plasma was stable for 7 days at −20 °C and 100 days at −70 °C. Our findings, showing a negative correlation between the levels of oxalogenesis (ΔOX) and initial OX concentrations, indicate that OX exerts an inhibition on the production of OX. Unsurprisingly, we also observed increased stability at lower temperature.

3.7. Evaluation of oxalogenesis in fresh samples

The effect of storage time at room temperature after sample collection on the *in vitro* oxalogenesis was tested in several ways. All samples were centrifuged without delay after collection, and the red blood cells discarded. First, plasma from seven healthy controls not taking any vitamin C supplement was collected. The plasma was divided into five aliquots. One aliquot was processed and analyzed immediately ($T = 0$) and the other four aliquots were left at room temperature and processed and analyzed after one, two, four and 24 h.

The results are shown in Fig. 3. As can be seen, the mean concentration of oxalate increases from a value of 6–49 μmol/L after 24 h. To test the effect of ingestion of vitamin C on the *in vitro* oxalogenesis, plasma from five healthy controls not taking any vitamin C supplement was collected. The plasma was divided into five aliquots and processed and analyzed using the same time protocol. The same healthy controls then ingested 1 g of vitamin C. After 2 h, plasma was collected and the experiment repeated. The results are summarized in Fig. 4. As can be seen, there was a significant higher mean increase (increased rate of oxalogenesis) in plasma oxalate values after ingestion of 1 g vitamin C.

These findings are in agreement with France et al. [16], but in contrast to Parkinson et al. [17].

In 1987, Winkler [18] demonstrated an inhibitory effect of EDTA and glutathione (GSH) on the rapid autooxidation of vitamin C, an effect attributed mainly to chelation of metal ions that catalyze autooxidation reactions.

Based on their findings, we wanted to test whether the addition of GSH to plasma, or even more simply collecting plasma in EDTA vials, could prevent the *in vitro* oxalogenesis.

First, a freshly made 1 mmol/L aqueous vitamin C solution was analyzed immediately after preparation (15 min) and after 22 h at room temperature, with and without addition of glutathione (GSH) or EDTA. Some of the vitamin C was converted to OX before the fresh sample was analyzed, but the increase in OX was negligible during 22 h storage at room temperature. When 100 μmol/L GSH or EDTA was added, the production of OX was immediately stopped (Table 2).

The effect of vitamin C on the stability of plasma, with and without addition of the inhibitors, was then tested (Table 3). A dramatic effect on the production of OX during 22 h storage was observed when 1 mmol/L vitamin C was added to plasma from a healthy control. As observed for the aqueous vitamin C solution (Table 2), the addition of GSH to plasma prevented the production of OX (Table 3), but the effect in plasma seemed to be time-limited as an increase similar to the untreated sample was observed from 3 to 22 h.

The use of EDTA vials for collection of plasma had no inhibitory effect on the production of OX (Table 3), but this could be due to the extremely high concentration of EDTA. Winkler [18] reported

Table 2
The concentration of OX (mmol/L) measured in an 1 mmol/L aqueous vitamin C solution immediately after preparation (0 h), and after storage at room temperature for 22 h, with and without addition of glutathione (GSH) and EDTA

	OX (μmol/L)		
	0 (h)	22 (h)	ΔOX
Vitamin C	16.6	19.6	3
Vitamin C + 100 μmol/L GSH	1.9	2.1	0.2
Vitamin C + 100 μmol/L EDTA	2.5	1.8	−0.7

The data are mean values of four replicates. ΔOX is the difference in OX concentration between 22 and 0 h.

Table 3
Oxalogenesis in heparin plasma at room temperature after addition of vitamin C and glutathione (GSH), and in EDTA-plasma

	OX (μmol/L)			
	0 (h)	3 (h)	22 (h)	ΔOX
Plasma	4.9	10.8	35.8	30.9
Plasma + 1 mmol/L vitamin C	12.1	24.1	205.5	193.4
Plasma + 100 μmol/L GSH	6.8	6.0	29.3	22.5
EDTA-plasma	10.4	17.2	76	65.6

ΔOX is the difference in OX concentration between 22 and 0 h.

a concentration dependence on the effect of GSH as inhibitor, and optimum protection at 100 μmol/L. They also reported similar protection using comparable low concentrations of EDTA and GSH, suggesting that the two inhibitors act by similar mechanisms. When collecting plasma in EDTA vials, the vacutainers contain 1.8 mg anhydrous EDTA per 1 mL blood (6.16 mmol EDTA per L blood). If the effect of EDTA as inhibitor shows similar concentration dependence as for GSH, the concentration of "free EDTA" in EDTA-plasma probably by far exceeds the optimal concentration.

In addition, EDTA interfered with the assay causing high background noise and poor peak shape.

Further investigations into the underlying mechanism, and the role of vitamin C on *in vitro*-oxalogenesis, were beyond the scope of this work but our observations show that an erroneously high plasma OX can be measured in plasma samples from healthy individuals taking vitamin C supplement unless the samples are correctly processed.

To test the effect of the endogenous level of OX on *in vitro* oxalogenesis, samples from before haemodialysis (expected to have high OX level) were collected from a PH1-patient. The samples were processed and analyzed at storage times of 0 h (endogenous level) and 24 h. The endogenous pre-dialysis concentration of plasma OX was 40 μmol/L, increasing to 76.2 μmol/L after 24 h at room temperature. Thus, the absolute increase in OX observed was lower for the PH1-samples with high endogenous OX levels, compared to the healthy controls.

Our results are in agreement with the findings of Parkinson et al. [17], who found that samples from patients with chronic renal failure (with high plasma OX) were much more stable at room temperature than plasma from healthy controls.

4. Conclusion

The reported SPE-LC–MSMS method provides adequate performance characteristics for the routine determination of OX in human plasma. Its test properties as a diagnostic tool for PH require a full evaluation with respect to sensitivity, specificity, positive and negative predictive values: however this was outside the scope of this article.

The most critical step in the assay was the second evaporation step. To minimize the loss of analyte during sample preparation, and ensure good MSMS signal intensity, quantitative removal of excess derivatization reagents should be performed under mild evaporation conditions. Pre-analytical factors, like delayed processing of plasma, can result in *in vitro* oxalogenesis, especially in samples from patients taking vitamin C supplements. Therefore, to minimize the risk of measurement of erroneously high plasma OX, samples should be processed within 1 h after collection, and stored at −70 °C for no more than 1 week.

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