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

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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

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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,