Liquid chromatography-tandem mass spectrometry quantification of oxalic acid in plasma and urine. Clinical implications in kidney transplant patients.

Doctoral Thesis by

Katja Benedikte Prestø Elgstøen

Department of Medical Biochemistry
and
Department of Medicine,
Oslo University Hospital Rikshospitalet
Oslo, Norway

Institute of Clinical Biochemistry
University of Oslo
Oslo, Norway

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"mamma leverer doktorgraden" Katinka 5 år

List of papers

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Scand J Clin Lab Invest. Accepted for publication

Abbreviations

ADH	alcohol dehydrogenase
AGT	alanine:glyoxylate aminostransferase
ALDH	aldehyde dehydrogenase
CE	capillary electrophoresis
C18	octadecyl carbon chain
C8	octyl carbon chain
Da	dalton, atomic mass unit
DAO	D-amino-acid oxidase
DGDH	D-glycerate dehydrogenase
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionization
ESRD	end-stage renal disease
ESRF	end-stage renal failure
FID	flame ionization detection
GO	glycolate oxidase
GC	gas chromatograph, gas chromatography
GC-MS	gas chromatography-mass spectrometry
GR	glyoxylate reductase
GFR	glomerular filtration rate
GGT	glutamate:glyoxylate aminotransferase
IMER	immobilized enzyme reactors
Ka	acid dissociation constant
LDH	lactate dehydrogenase
LC	liquid chromatography
LC-MSMS	liquid chromatography – tandem mass spectrometry
MRM	multiple reaction monitoring
MS	mass spectrometer, mass spectrometry
MSMS	tandem mass spectrometry
m/z	mass-to-charge ratio
NAD	nicotinamide adenine dinucleotide (oxidized: NAD^+ and reduced: NADH)
OF	Oxalobacter formigenes

PAT	putative anion transporter
PCR	polymerase chain reaction nb sjekk i tekst
PH	primary hyperoxaluria
PH1	primary hyperoxaluria type 1
PH2	primary hyperoxaluria type 2
pKa	- $\log_{10}$ Ka (Ka : acid dissociation constant)
RRT	renal replacement therapy
SIRE	sensors based on injection of the recognition element
SLC	solute-linked carrier
SPE	solid phase extraction
TX	transplantation
u	atomic mass units
UV	ultraviolet

Errata

Paper I

In the *Introduction* section it should read ”Being a strong acid (pK_{a1} 1.2**7** and pK_{a2} **4.28**), OX was therefore expected to be suitable for **SPE with SAX material**.”

In Table I, it should read ”Current procedure, LC-MSMS **3-11** 67 ”

In the *Result and discussion* section, under *3.1 SPE-LC-MSMS method development* it should read “….sample loss; and the evaporation and **chromatographic**-separation times required…”

In the *Result and discussion* section, under *3.7 Evaluation of oxalogenesis in fresh samples* it should read “As can be seen, the mean concentration of **OX** increases from a value of 6 **to** 49 μmol/L….”

Introduction

1. Oxalic acid, hyperoxaluria, hyperoxalaemia and oxalosis

Oxalic acid or oxalate is a small dicarboxylic acid with molecular mass 90.03 g/mol.
It is a relatively strong acid with pKa_1=1.27 and pKa_2= 4.28.
It is the physical property of its calcium salt which is very insoluble at physiological pH that is responsible for the life-threatening property of oxalic acid in humans[1].

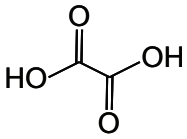


Figure 1. Oxalic acid

The terms *hyperoxaluria* refers to increased urinary excretion of oxalic acid , *hyperoxalaemia* refers to increased concentration of oxalate in blood, and the term *systemic oxalosis* or simply *oxalosis* refers to the accumulation of oxalic acid in tissues[2].
There are several different causes of hyperoxaluria, -aemia and oxalosis, and many of the causes are interactive. If the body burden of oxalate increases, hyperoxaluria will be the consequence. Hyperoxalaemia occurs when the body's ability to excrete oxalate through the kidneys decreases, and oxalosis can be the consequence of long term or extensive hyperoxalaemia. Hyperoxaluria/aemia increases the risk of precipitation of calcium oxalate crystals in the kidneys (renal oxalosis) and consequently the formation of calcium oxalate kidney stones.
Environmental or *secondary* hyperoxaluria can be caused by increased ingestion and/or absorbtion of dietary oxalic acid, intestinal disease or surgery or alterations in intestinal flora. Increased endogenous oxalic acid production, e.g. by ethylene glycol poisoning, can also result in secondary hyperoxaluria/oxalosis. Finally, decreased clearance of oxalic acid from the body due to renal insufficiency can result in hyperoxalaemia and consequently oxalosis.
Hyperoxaluria/oxalosis is only infrequently caused by genetic factors; the *primary* hyperoxalurias. The different topics will be discussed separately.

2. Biochemistry and metabolism

Oxalic acid is a metabolic end product that is excreted almost solely in urine.
In the body, oxalic acid is derived from two sources: the ingestion of oxalic acid or ingestion of precursors of oxalic acid, that is, compounds that are metabolized into oxalic acid in the body. In the following oxalic acid from food is referred to as dietary oxalic acid and oxalic acid derived from metabolism of oxalic acid precursors as endogenous oxalic acid. From an analytical point of view, an additional source of oxalic acid is of major interest: The non-enzymatic conversion of ascorbic acid (vitamin C) into oxalic acid, known as oxalogenesis can lead to erroneously high oxalic acid quantified in body fluids.
The different sources of oxalic acid will be discussed separately.

Dietary oxalic acid

There is considerably controversy surrounding the various factors determining the concentration of oxalic acid in urine [3]. In 1995, Holmes et al estimated that a healthy diet rich in whole grain products, vegetables and fruit may contain close to 200mg of oxalate pr day, while a less healthy diet, rich in animal protein, refined sugar and fat may contain less than 100 mg of oxalic acid, and that 5-15% of the dietary oxalate is absorbed in the intestine depending on co-ingestion of calcium, magnesium and fiber, the latter apparently due to reduced transit time [4]. The contribution of dietary oxalate to oxalate excreted in urine has been reported to be as high as 50% [5]. Many different foods contain oxalate. The high oxalate content of dark-green leafy vegetables like spinach and rhubarb is generally known. Consumption of 200g boiled rhubarb or spinach has been reported to increase urinary excretion of oxalate by 300-400% [6]. However, despite high in oxalate content, it is important to take into account the relatively low amount of consumption of especially rhubarb. Other sources of dietary oxalate like black tea and cocoa (chocolate) may contain only moderate amounts of oxalate, but the daily consumption in many individuals might be comparably higher. The oxalate content of vegetables depends on the plants maturity and age, soil quality, and climate, complicating the accurate assessment of the amount of oxalate ingested in diets. As an example, using capillary electrophoresis (CE), the oxalate content of sweet potato purchased at three different occasions at the same supermarket was found to vary from 0.2 to 86.9 mg oxalate/100g[7]. Difficulties in obtaining reliable data of the oxalate content of food is also hampered by the fact that different analytical methods are used in

different studies[8;9]. Food oxalate analysis is challenging due to the wide range of interfering substances present in addition to both the potential loss and generation of oxalate during assay [9]. Foods contain oxalate in both soluble and insoluble forms, and the ratio of the two may have influence on the intestinal absorption and bioavailability of oxalate from different food sources. Using different extraction conditions for total and soluble oxalate, Honow et al [9] developed a quantitative method for oxalate in foods by anion exchange chromatography and detection using an enzyme reactor with oxalate oxidase. The oxalate content of about 150 foods has been established using their methodology, some of which are found in Table 1.

Table 1. oxalate content of some foods [8;9]

Food	oxalate content mg/100g		Food	oxalate content mg/100g	
	Soluble	Total		Soluble	Total
rhubarb	380	570-1900	white bread	4.9-8.6	
spinach [a]	33.3-168	100-627	apple	0.3-1.8	0.4-5.8
potatoes [a]	8.8-18.9	8.8-35.3	banana	0.1-2.2	0.1-2.2
tomato	2.5-4.5	3.7-13.7	orange	0.2	1.8
beans	1.5	13.9	rye bread	0.9	
asparagus [a]	0.5-1.1	1.8-3.1	black tea	2.5-6.2	
broccoli [a]	0.5-1.7	0.8-1.9	cocoa powder		154-980
carrot [a]	2.3	4.9	Beer		1.7-1.8
rice [a]	0.4	1.8	Coffee	0.5-0.7	
raspberry	2.7-5.9	11.3-25.7	apple juice	0.07-0.35	0.8-0.9
carambola	81.4-185.6	212.6-345.7	1 bar with chocolate	7.1	37.9
strawberry	0.6-1.9	1.5-4.3			

[a] Cooked

The oxalate content of the foods ingested will be only one of several variables that influence the amount of oxalate absorbed. Other factors will include the bioavailability of the ingested oxalate, the amounts of oxalate-binding cations, the inherited capacity to absorb oxalate, the transit time in the small and large intestines, and the activity of oxalate-degrading bacteria in the large intestine [7].

Bioavailability of oxalic acid

The bioavailability of oxalate in foodstuff depends on its interaction with other components of food, especially calcium[10]. Mixing oxalate containing foods with diary products can reduce the amount of oxalate available for absorption, presumably because calcium in the diary products precipitates oxalate, and calcium oxalate crystals formed might not at all be redissolved under normal gastric conditions [3].

Soluble oxalate has generally been thought to have higher bioavailability than insoluble oxalate. However, a study on the bioavailability of oxalate from oca (*oxalis tuberosa*), a vegetable containing oxalate in soluble form only, was found to be in the same range (1.44%) as for spinach (2.44%), containing both soluble and insoluble oxalate. A large variation in oxalate uptake from oca among individuals was also found. Still, a reduced uptake of
oxalate from oca was found when the vegetable was consumed with sour cream (containing calcium) [11].

Historically, calcium oxalate stone formers have been advised to convert to a vegetarian diet, as the urinary excretion of oxalate was suggested to decrease with a low intake of animal proteins (containing oxalate-precursors). However, more recent results show that a vegetarian diet results in an increase in oxalate excretion [10;12]. The higher urinary oxalate excretion in vegetarians most likely is due to an increased ingestion of vegetables rich in oxalate.

Recent results from Thomas et al[13] on the dietary influence of intestinal oxalate absorption and excretion in healthy volunteers confirmed the increase in oxalate excretion found with a vegetarian diet. They also tested the effect not only on vegetarian vs mixed diet, but also between low and high oxalate vegetarian diet (70 and 300 mg oxalate/day respectively) that was equal in calcium and other nutrients and fluids. Surprisingly, no significant correlation in oxalate absorption with dietary oxalate was found. However, this unexpected finding was recognized to stem from the fact that although the calcium content of the low and high

oxalate diets used in the study was the same, the calcium content of the single meals varied thus influencing the bioavailability of oxalate from each meal (tortellini with spinach in cream sauce used in the high oxalate-diet). These findings support the view that attention must be paid to not only having an adequate daily intake of calcium but also on the timing of ingestion in relation to the timing of consumption of foods rich in oxalate[13].
Although oxalate excretion is higher following a vegetarian diet, the calcium excretion is lower, even when comparing mixed and vegetarian diets with equal amounts of calcium. Calcium excretion is affected by animal protein levels (higher excretion following ingestion of animal proteins) resulting in an overall reduced risk of supersaturation and stone formation in this section of the population [12].

Absorption of oxalate

Traditionally, the absorption of oxalate has been investigated by use of the radioactive $^{14}C_2$ oxalic acid in the so called isotopic method, the use of load method or daily excretion method as reviewed by Holmes et al[4]. More recently, Voss et al described a harmless $^{13}C_2$ oxalic acid absorption test as alternative to the use of radioactive oxalate[14].
Oxalate absorption can occur along the entire gastrointestinal tract [4]. By administrating an oxalate load with $^{13}C_2$ oxalic acid and measuring the concentration of this isotopically labeled oxalate in urine in the following eight hours, a maximum peak of absorption was found 2-4 hours post load, which is compatible with absorption in the small intestine[15]. However, these studies of Knight et al also suggest that some individuals (stone formers and non-stone formers) have an enhanced absorption in the large intestine. The identification of the SLC (solute-linked carrier) gene superfamily has further helped in unrevealing the mechanism of intestinal oxalate handling. Several structurally similar proteins encoded by the SLC26 gene family are anion transporters having measurable affinity for oxalate and are expressed in the intestine. The importance of the anion transporters in oxalate homeostasis has been demonstrated by the use of a knock-out mouse model. In SLC26c6 (coding for the putative anion transporter PAT-1) knockout mice, the ileal oxalate absorption and subsequent urinary oxalate excretion was shown to be enhanced [16]. The complex roles and mechanisms of intestinal oxalate transport in oxalate homeostasis has recently been reviewed by Hatch et al[17].
Several members of the SLC26 family are expressed in the kidney as well[18], and their role in renal anion transport is briefly discussed under “renal handling of oxalate”.

Oxalate degrading bacteria in the gastro intestinal tract

Oxalobacter formigenes (OF) is an anaerobic bacterium that relies exclusively on metabolism of oxalate in the colon for energy. Colonization of the gut with OF has been associated with a reduction in risk of recurrent stone disease and significantly lower urinary oxalate excretion [19] [20]. In fact, a single oral dose of OF has been shown to reduce oxalate excretion in healthy adults administrating an oxalate load [21]. A robust colonization with OF has a degrading capacity of up to 1g (11.1mmol) of oxalate/day in the human gut [22]. Thus, using of OF's complete dependence of oxalate is at least a theoretical potential tool to prevent recurrent stone disease and reduce hyperoxaluria.

OF produces formate and CO_2 (carbon dioxide) as an end product of metabolism. The membrane of the bacterium contains an oxalate^{2-} formate^{1-} antiporter that mediates the entry of oxalate and export of formate. Two enzymes are involved in the metabolism of oxalate into CO_2 and formate: formyl-CoA transferase (transferring Coenzyme A from formyl-CoA to oxalate) and oxalyl-CoA decarboxylase (decarboxylating oxaloyl-CoA to formyl CoA plus CO_2). CO_2 then diffuses out of the cell [23]. Using a polymerase chain reaction (PCR)-based detection assay Sidhu et al [24] determined the presence of OF in the gastrointestinal tract of healthy children. A complete absence of OF in newborns and infants up to 6-9 months was found, but the bacterium appeared when children reach about one year old, indicating that colonization starts when children start crawling about. By 3 to 4 years of age, all children were colonized with OF, declining to the adult level of 60-70% between 8 and 12 years [24]. The reason for the loss of OF is not clear, but the therapeutic use of antibiotics may play an important role [23]. In fact, the prevalence of colonization of OF has been shown to be lower in both healthy individuals and recurrent calcium oxalate stone formers who have used antibiotics to which OF is sensitive at any time in the past[25]. Oxalate secretory pathways for extra-renal oxalate elimination have been identified, and it has been hypothesized that OF can contribute to maintain a transepithelial gradient favouring passive oxalate movement from blood to the intestinal lumen [26].

Endogenous oxalate

Approximately 10 to 20 mg of oxalate is produced in an adult human every day, and it is widely assumed that the main source of endogenously produced oxalate in humans is the liver [27]. The major precursor of oxalate is sugars and amino acids, and about 40 percent of oxalate synthesis appears to be derived from glycine metabolism [2].

There are still some unresolved issues in the biochemical reactions in human cells that culminate in the synthesis of oxalate [27 2 28]. However, the biochemical hallmarks and the enzyme deficiencies involved in the metabolic diseases covered in this text, the primary hyperoxalurias, are well known. Figure 2 shows the major reactions involved in glycolate, glyoxylate and oxalate metabolism in the human hepatocyte [2;29].

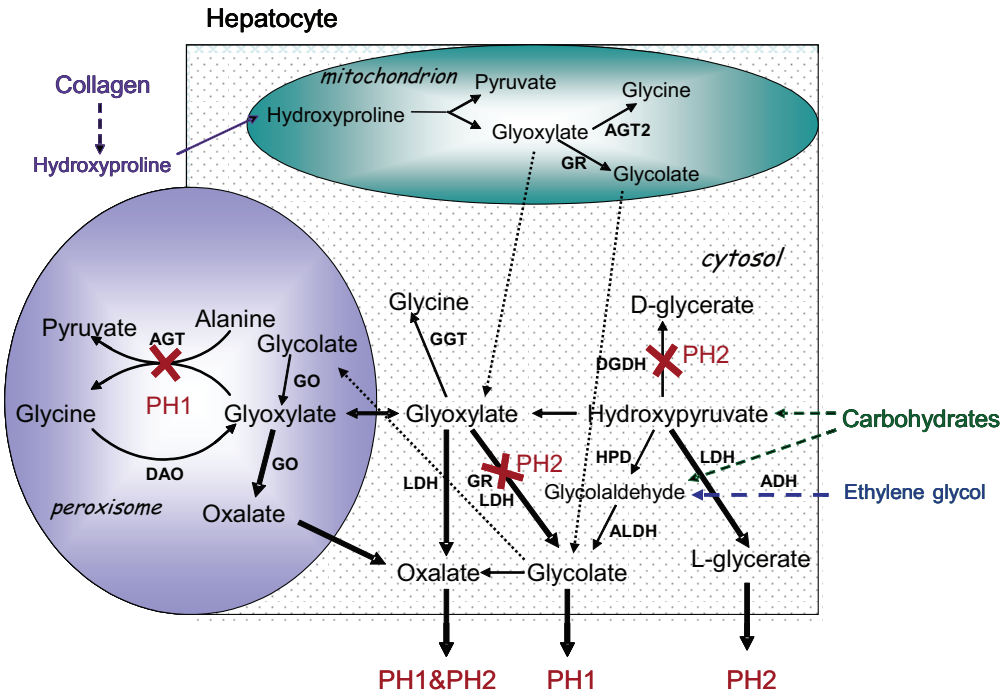


Figure 2. Major biochemical pathways involved in oxalic acid metabolism in the human hepatocyte.

Most oxalate precursors are metabolized via glycolate and/or glyoxylate. Immediate precursors of the highly reactive glyoxylate are glycolate and glycine. Glycolate is oxidized into glyoxylate by glycolate oxidase (GO) or L-2-hydroxy acid oxidase A (not shown), while the oxidative deamination of glycine to glyoxylate can be catalyzed by D-amino-acid oxidase (DAO) or glycine oxidase (not shown). Detoxification of glyoxylate by transamination into glycine is catalyzed by alanine:glyoxylate aminotransferase (AGT) in peroxisomes. In cytosol, the deamination into glycine is catalyzed by glutamate:glyoxylate aminotransferase (GGT). In human, AGT is liver-specific while GGT activity is widely dispersed. In cytosol, glyoxylate can also be reduced to glycolate by lactate dehydrogenase (LDH) or glyoxylate reductase (GR) which is also widely dispersed. Oxidation of glyoxylate into oxalate is catalyzed by GO in peroxisomes and by LDH in cytosol.
Glycolate is an important precursor of glyoxylate, but the sources of glycolate has not been fully identified. Experiments with collagen ingestion suggests that the metabolism of hydroxyproline (an amino acid of collagen), principally occurring in mitochondria of hepatocytes and renal proximal tubule cells may be of importance[30]. AGT 2, converting glyoxylate to glycine, has no homology with AGT1 and is found in mitochondria of most tissues[28]. It has also been shown that the main route by which carbohydrates such as glucose and fructose are converted to oxalate is through hydroxypyruvate[31-34]. Hydroxypyruvate is converted to glycolaldehyde by hydroxypyruvate decarboxylase (HPD), an enzyme that is found in various tissues. The glycolaldehyde is then presumeably oxidized by aldehyde dehydrogenase (ALDH) into glycolate. In normal circumstances the in vivo relevance of the hydroxypyruvate-to-oxalate pathway is uncertain as most of the hydroxypyruvate would be expected to be reduced to D-glycerate catalyzed by D-glycerate dehydrogenase (DGDH)[2].

Ethylene glycol poisoning

Ethylene glycol is a common constituent of antifreeze and de-icing solutions, and ethylene glycol poisoning can result in acute renal failure and death[35;35;36]. The toxicity of ethylene glycol is linked to its metabolism to oxalate, initially via alcohol dehydrogenase (ADH) to glycolaldehyde. Glycolaldehyde is rapidly converted to glycolic acid resulting in the severe metabolic acidosis often found in ethylene glycol poisoning. Glycolic acid is slowly metabolized to oxalate that can precipitate as calcium oxalate in the kidney.
It is the calcium oxalate that is responsible for the renal toxicity of ethylene glycol.

As the conversion of ethylene glycol into it's toxic metabolites is catalyzed by ADH, the treatment of ethylene glycol poisoning targets the inhibition of this enzyme. Historically, ethanol has been used as an antidote as ADH has a higher affinity for ethanol than for ethylene glycol. Today, drugs like fomepizole that effectively blocks ADH are used for treatment of ethylene glycol poisoning.

Vitamin C

The potential effect of Vitamin C (ascorbate) on endogenous oxalate production and urinary oxalate levels is uncertain and investigation of endogenous oxalate production is difficult as this requires the ingestion of a diet entirely free of oxalate. However, by comparing the urinary oxalate excretion in calcium oxalate stone formers and healthy individuals on a totally controlled low-oxalate diet, with and without vitamin C supplement, the stone formers, but not the controls, were found to have increased endogenous oxalate production and secretion suggesting that vitamin C supplementation might be a risk factor for individuals that are predisposed to kidney stones [37]. A recent study on oxalate excretion following intravenous administration of large doses of ascorbic acid (0.2 to 1.5g/kg body weight) in subjects with normal renal function revealed that only about 0.2% of the ascorbic acid appeared as oxalate in the urine[38].

3. Renal handling of oxalate

In the nephron, oxalate is freely filterable at the glomerulus. Further processing of oxalate in the tubules may involve tubular reabsorption and secretion modifying the ultimate renal excretion of oxalate. However, despite a number of studies published on the renal handling of oxalate (e.g. [39;40]), relatively little is truly known due to technical difficulties in performing such studies (Professor Ross P. Holmes, Department of Urology, Wake Forest University School of Medicine, Winston-Salem,USA, personal communication).

Tubular reabsorption and secretion

According to Robertson[41] oxalate is reabsorbed in the early proximal tubules. Transcellular reabsorption of oxalate depends on anion exchangers, and several members of the multifunctional anion exchanger family SLC26 are expressed in the kidneys[18]. In proximal tubules the transporter SLC26a1 is responsible for sulfate and oxalate transport.
Verkoelen et al[42] concluded after reviewing published reports that oxalate is also actively secreted in the proximal tubule. Also according to Robertson oxalate is secreted in the late proximal tubule [41]. Oxalate secretion is associated with the SLC26a6 is a Cl-/anion exchanger involved in proximal tubular sodium and chloride absorption with exchange for oxalate leading to net oxalate excretion [43;44].
Apart from kidney function determining the filtration of oxalate, the net renal excretion depends also on the degree of tubular reabsorption and secretion. These factors may not be constant over time. Actually the renal oxalate handling has been reported to vary with the amount of oxalate ingested (from reabsorption during fasting and secretion during high oxalate intake) indicating that after an oxalate rich meal the kidney could be secreting oxalate for an extended period of time[15].

Calcium oxalate stone formation

The first description of calcium oxalate crystals identified in urine dates back to 1839[45], but as early as in 1810 certain renal stones were found to contain calcium oxalate[46]. It is not surprising that salts may form crystals and stones in the renal tubules and urinary tract since the glomerular filtrate is up to 100- fold concentrated during the passage of the nephron with modest water intake.

The physiochemistry of stone formation in general is complex, and for calcium oxalate several mathematical models has been developed to describe the process [41]. However, it is generally agreed that the initiation and growth of a crystal involves a chemical precipitation from a solution that has become supersaturated with respect to stone-forming solutes as the glomerular filtrate traverses the nephron, and that factors increasing the transit time increases the risk of a crystal to become lodged at some point in the nephron [41;47-49]. Consequently, hyperoxaluria contribute to calcium oxalate stone formation simply by increasing the urinary saturation of calcium oxalate.

4. Primary hyperoxaluria

Primary hyperoxaluria (PH) includes two rare, well characterized autosomal reccesive diseases: primary hyperoxaluria type 1 (PH1) and primary hyperoxaluria type 2 (PH2) [2;29;50]. In addition, a third type of primary hyperoxaluria has been suggested [51;52].

The incidence of PH1 is 1:120 000 live births in Europe [53]. Less attention has been paid to the elucidation of PH2, probably due to its even greater rarity than PH1. PH2 is often considered to be a milder disease than PH1 and a ratio of PH1 to PH2 of 20:1 has been estimated[54].

PH is characterized by overproduction and accumulation of oxalate in tissues. The first identification of PH was published in 1925[55], but it took another 25 years before PH was first described in detail in a report of oxalosis in a child who died of renal failure[56]. A few years later, in 1954, the familial nature of the disease was emphasized with a report of oxalosis and hyperoxaluria in identical twins [57].

The main elimination of oxalate from the body is by urinary excretion, resulting in the characteristic increased urinary concentration of oxalate found in PH. The excess oxalate binds to Ca^{2+} and forms insoluble calcium oxalate that deposits in the kidney and urinary tract.

To expand knowledge of PH by accumulating information regarding a larger number of patients, Lieske et al[58] at Mayo Clinic College of Medicine, Rochester, Minn., USA have developed an international registry for PH that can be found at[59]:
http://mayoresearch.mayo.edu/mayo/research/nephrology/registry.cfm

By April 2009 (latest update available on the website) 203 patients had been registered.

PH1

PH1 is caused by deficiency of the liver specific peroxisomal enzyme AGT (see Figure 2). Approximately one-third of the PH1 patients have significant levels of AGT catalytic activity, but a unique intracellular protein trafficking defect result in AGT being located in mitochondria instead of peroxisomes.

AGT deficiency has a major impact on glyoxylate detoxification, and failure to detoxify glyoxylate within the peroxisomes results in either more glyoxylate being oxidized into oxalate by GO or more glyoxylate diffusing into the cytosol. Once in the cytosol, glyoxylate

can be oxidized to oxalate by LDH, transaminated to glycine by GGT or reduced to glycolate by GR or LDH. The resulting excessive hepatic production of oxalate and glycolate is the biochemical hallmark of PH1.

In PH1, more than 90% of the cases present with symptoms referable to the urinary tract, and the most common is calcium oxalate stone disease[29].

According to Cochat et al [53], PH1 typically fits five presentations:

1) an infantile form with early nephrocalcinosis and kidney failure
2) recurrent urolithiasis and progressive renal failure leading to a diagnosis of PH1 in childhood or adolescence
3) a late onset form, with occasional stone passage in adulthood
4) diagnosis given by post-transplant recurrence
5) pre-symptomatic subjects with family history of PH1 (usually siblings)

A number of case reports have been published on patients presenting with recurrent urolithiasis or end stage renal failure who later become diagnosed with PH1[60-62]. Case reports describing the extremely rare severe infantile form of PH1 presenting as a life threatening condition with end stage renal disease (ESRD) and nephrocalcinosis in a 3-month old baby[63] and the late onset form presenting as chronic pain in both hands in a 61 – year old man[64] are examples on the enormous spread in the ages at which the disease become apparent.

PH 2

PH 2 is caused by a deficiency of the cytosolic enzyme DGDH/GR (see Figure 2). The conversion of hydroxypyruvate to D-glycerate catalyzed by DGDH is heavily weighted towards the reduction reaction. Thus the biochemical consequence of the enzymatic defect in PH2 is a buildup of hydroxypyruvate that instead is reduced to L-glycerate by LDH. L-glycerate is normally not detectable in urine. The exact mechanism of excessive oxalate synthesis in PH 2 is not known, and several hypotheses have been advanced. Still, the fact that DGDH and GR are different catalytic activities of the same enzyme is regarded the most plausible explanation of hyperoxaluria found in PH2. The lack of GR activity in PH2 is thought to prevent the reduction of glyoxylate to glycolate with the subsequent conversion of excess glyoxylate to oxalate by LDH[65].

Clinical and biochemical diagnosis of PH

In PH, progressive deposition of calcium oxalate often leads to deteriorating kidney function and finally ESRD [2;29].The kidneys ability to excrete oxalate then drops, and the plasma concentration increases. Supersaturation with respect to calcium oxalate occurs when the plasma concentration of oxalate reaches approximately 40 μmol/L in adults[66] resulting in deposition of calcium oxalate in other organs, especially bones. This systemic oxalosis is a common finding in PH. There is an average 5-year time interval from symptom onset to diagnosis of PH[58]. The rarity of the disease and insufficient knowledge about inherited urolithiasis is thought to be the explanation of this delayed diagnosis of PH[53]. Stone forming activity in PH2 is lower than in PH1 and systemic oxalosis exceptional. However, myocardial oxalosis in a PH2 patient has been reported [54].

The accumulation of calcium oxalate in PH patients starts when the renal function is only slightly impaired and the resulting systemic oxalosis is associated with pathology according to the tissue concerned, e.g. bone pain when deposition of calcium oxalate is within the bone.

Biochemical findings

PH is most commonly diagnosed by measuring oxalate excretion, and the oxalate excretion in PH is in general grossly elevated. In addition, increased urinary glycolic acid in PH1 and L-glyceric acid in PH2 are normally found. However, in PH1 the increased concentration of oxalate in body fluids is not always associated with increased concentration of glycolic acid. Differences in relative contributions made by the different enzymes involved in oxidation and reduction of glycolic acid (GO, LDH and GR) is a possible explanation for the considerable biochemical heterogeneity in PH1 with respect to the ratio of glycolic acid and oxalate excreted in urine.

In PH, when glomerular filtration rate (GFR) falls below 30-50ml/min pr 1.73 m^2 systemic oxalosis starts to occur [53] (see “Kidney failure and hyperoxalaemia” on page 36 for explanation of GFR). The major compartment of the insoluble oxalate pool is bone, and the bone content of oxalate has been reported to be much higher in PH1 patients than in patients with ESRD due to other causes. [67].

Concomitant with the decreasing urinary excretion of oxalate following renal insufficiency is the increase in plasma oxalate.

Thus, the biochemical findings in PH vary during the course of the disease (See Figure 3). With sufficient renal function, a normal or close to normal plasma level of oxalate combined with a grossly elevated urinary oxalate excretion are the typical laboratory findings. However, significantly increased plasma concentration of oxalate has been reported in children with PH despite even with a normal kidney function[68]. With deteriorating kidney function, oxalate excretion can drop to normal values while the plasma concentration increases dramatically. As a consequence, analysis of oxalic acid in both plasma and urine are important in laboratory diagnosis of PH.

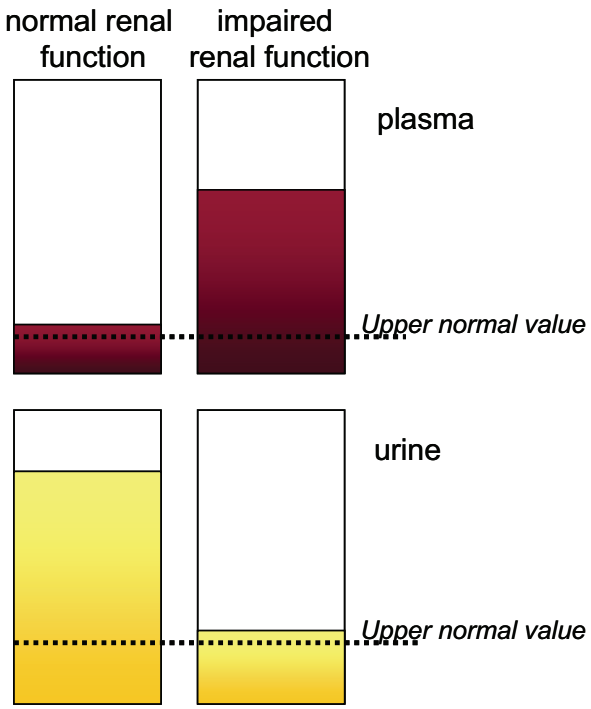


Figure 3. Dependence of kidney function on the biochemical findings in PH.

Enzymatic diagnosis

Differential diagnosis between the two subtypes of PH is essential if liver transplantation is considered, as the defect in PH1 is a liver specific enzyme and in PH2 the deficient enzyme is widely dispersed. For definite diagnosis of PH1, measurement of the activity of AGT in liver needle biopsies can be performed [2], and by immunoelectron microscopy the subcellular distribution can be determined [69]. There is no clear relationship between clinical severity and residual AGT catalytic activity[62]. For definitively diagnosis of PH2, measurement of the GR activity in a liver biopsy has traditionally been performed although the deficient enzyme in PH2, DGDR/GR, is expressed in virtually every tissue in the body. As a result, the utilization of more readily available cells for enzymatic diagnosis of PH2 has been suggested. A relatively simple assay for spectrophotometric quantification of GR and DGDR activity in blood mononuclear cells has been described that could potentially be used as a minimally invasive diagnostic test for PH2[70;71].

Molecular genetics of PH

The deficient enzyme in PH1, AGT, is encoded by a single gene, AGXT, and the gene comprised of 11 exons, spanning approximately 10kb, and maps to chromosome 2q37.3[72]. A total of 146 mutations scattered across the gene have been described, with all exons represented. Major or minor deletions and insertions account for 25% of the mutations, while the majority (75%) is point mutations [73]. The first mutation to be described and also the most common is a Gly170Arg replacement which is found in about 30% of PH1 mutant alleles [74]. This common mutation enhances the strength of a functionally weak mitochondrial targeting sequence generated by a Pro11Leu polymorphism and together, the mutation and the polymorphism are responsible for the peroxisome-to-mitochondria mistargeting of AGT[75].

PH2

The deficient enzyme in PH2, GR/HPR, is encoded by the GRHPR gene with 9 exons, spanning 9kb, and maps to chromosome 9[76]. Fifteen mutations spread throughout the nine exons have been described [77].

Treatment of PH

Following the general order of disease progression, the first strategy in the treatment of PH is to reduce the amount of oxalate in the body. Dietary restrictions in intake of oxalate-containing foods is not regarded as being very efficient in PH[62], but reduction of oxalate absorption by co-ingestion of calcium can at least theoretically reduce the dietary oxalate contribution to the total corporeal oxalate load[2]. The ability of the bacteria OF to both stimulate secretion of endogenous produced oxalate and degrade it in the intestine has been proposed as a potential tool for the treatment of PH1 [22].

By orally administrating OF for 4 weeks as frozen paste or enteric-coated capsules (delivering OF past the very acidic conditions in the stomach) to a total of 16 PH patients, a marked reduction in urinary oxalate or plasma oxalate was observed in the majority of patients. In addition, two of the PH patients with systemic oxalosis reported amelioration of clinical symptoms during OF therapy [22]. The findings of Hoppe et al[22] has suggested applicability of OF treatment of PH patients at all stages of the disease, but especially in those who are on maintenance dialysis and in renal failure[78].

Many strategies to normalize endogenous oxalate production in PH by reduced intake of oxalate precursors or inhibition of the enzymes involved in the production of oxalate (see Figure 2) have been proposed, but few has reached general acceptance and use[2].

The role of hydroxyproline derived from meat and gelatin has been given some attention in recent years as it has been estimated that up to 20% of the endogenously produced oxalate excreted in urine is derived from metabolism of hydroxyproline through the glycolate – glyoxylate –oxalate pathway[28;30;79].

Although most attempts at treatment by metabolic intervention and pharmacologic manipulation has had limited success, the administration of pyridoxine (vitamin B6) is an exception. The effect of pyridoxine in decreasing urinary oxalate excretion in some, but not all, PH patients has been known for almost 50 years[80]. Although attributed to its role as a cofactor of AGT, the molecular basis concerning the mechanism of action of pyridoxine still remains unknown. The predictability of response has also been largely unknown but recent findings indicate that two mutations resulting in peroxisomal-to-mitochondrial mistargeting of AGT are associated with pyridoxine response [81]. Following these findings, genotyping to predict pyridoxine response has been suggested [82].

If the urine oxalate concentration does not normalize following the strategy mentioned above attempting to reduce total body oxalate burden, the second strategy for the treatment

of PH is the prevention of calcium oxalate crystallization by hydration or use of crystallization inhibitors. If prevention of crystallization fails, the agglomerations may be removed by lithotripsy or open surgery, or if renal failure develops the third strategy will be dialysis or kidney transplantation[2]. Clinically the patients present at all stages of the disease, thus the order of implementation of treatment strategies in PH varies.

Organ transplantation in PH1

Early diagnosis and intensified conservative treatment is the main goal in PH, but if unsuccessful, several transplantation (TX) strategies are available [83].

Kidney transplantation alone does not cure the disease, but rather attempts to recover from the consequences of lost kidney function and not the basic defect in the liver of PH1 patients. Following single kidney transplantation, the endogenous oxalate synthesis therefore remains elevated. Although in some cases the transplanted kidney can survive for a significant length of time, poor prognosis due to recurrence of oxalosis in the graft has been well documented and particularly with deceased donor grafts. [61; 84-86].

Liver transplantation can be regarded as a form of gene therapy as well as enzyme replacement therapy as it will supply the missing enzyme in the correct organ (liver), cell (hepatocyte) and cell compartment (peroxisome). However, liver transplantation as a form of gene therapy is far from ideal as it involves the replacement of thousands of perfectly normal genes just to replace the one that is abnormal. As the function of the liver is normal except from the missing AGT activity in PH1 patients, the liver harvested from such a patient has been used as a donor organ for a subsequent graft in a second liver recipient in a so-called domino procedure. As could be expected, the domino liver recipient rapidly developed hyperoxaluria as PH1 in this case was transferred from the donor to the recipient [87].

The concept of curing the metabolic defect in PH1 before renal damage occurs by performing preemptive liver transplantation has received considerable attention. Thus, for prevention of ESRF and treatment of PH1, preemptive liver transplantation is regarded as a powerful tool, however ideal timing and patient selection is regarded difficult as the risk associated with surgery and long-term immunosuppression have to be weighed against complications related to oxalosis and morbidity[88].

For PH1 patients with advanced renal insufficiency or ESRD, combined liver/kidney transplantation has the advantage of not only correcting the underlying metabolic defect but also replacement of renal function [60;85;89]. Combined liver/kidney transplantation can be performed concurrent (simultaneous) or sequentially (first liver, then kidney)[89].

The rate of endogenous oxalate synthesis would be expected to drop to normal levels immediately after combined liver/kidney transplantation, but it may take months or years to normalize the urinary oxalate excretion, depending on the time span of renal insufficiency and subsequent oxalate pool size built up prior to transplantation[60;85]. The accessibility of the calcium oxalate stores to the blood stream will influence on the resolubilization rate, and thus deposits in slow-turnover bone would be expected to be slowly dissolved. In one patient with huge stores of oxalate accumulated in the skeleton prior to transplantation, the urinary oxalate excretion was found to drop until month 7 post transplantation but reascended in the following months[90], probably due to resolubilization of the oxalate stored in bones.

At Oslo University Hospital Rikshospitalet, combined liver/kidney transplantation was performed in two PH1 patients in the late 80-ies. They were between 20 and 30 years of age at that time. Both patients have been retransplanted with kidneys 4 times. It appears that the body load of oxalate is so huge in adult patients that kidney failure occurs in transplants also after correction with a liver transplantation. This argues in favor of early liver transplantation, especially since the prognosis has improved substantially over the last years[91]

In Figure 4, the predicted effects of kidney alone and combined kidney/liver transplantation on oxalate synthesis, urinary excretion, plasma concentration and total body burden of oxalate shown.

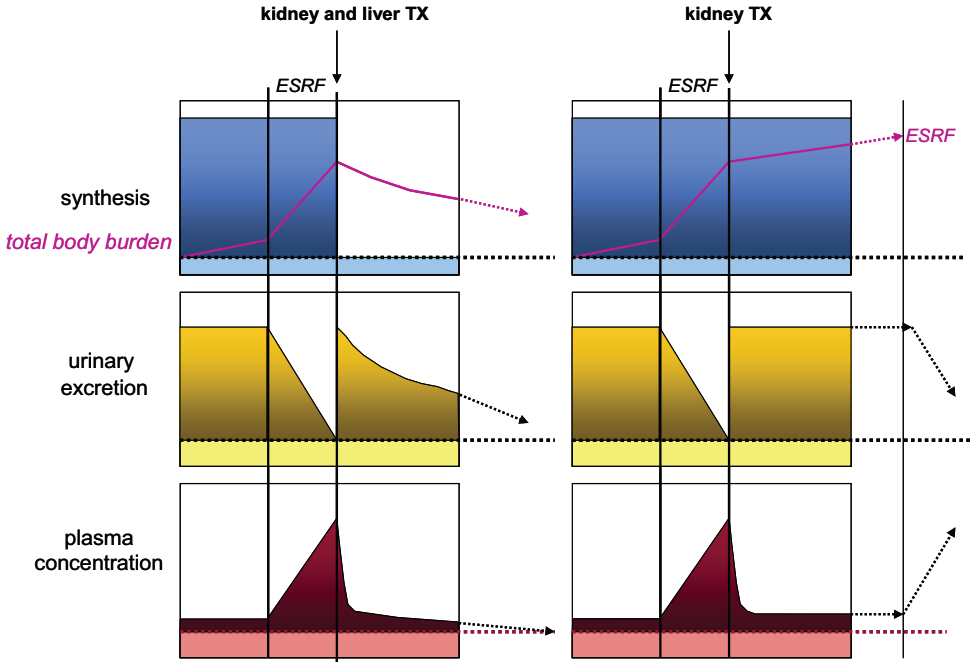


Figure 4. Effect of kidney alone and combined kidney/liver transplantation on oxalate dynamics in PH1. Dotted line represents upper normal level.

In PH2, liver transplantation has not been reported.

This is probably due to the fact that PH2 is regarded as a milder disease than PH1, and in addition the liver must contain a significant proportion of the body's requirement for the missing enzyme, GR/DGDH, for liver transplantation to work as an enzyme-replacement therapy[2]. The tissue distribution of GR/DHDG is uncertain, but it has been shown that the liver may contain much of the body's GR activity [92].

However, isolated kidney transplantation in PH2 has been reported[93].

5. Secondary hyperoxaluria and/or hyperoxalaemia

As mentioned earlier, secondary hyperoxaluria is due either to excessive dietary oxalate intake or increased intestinal oxalate absorption. Secondary hyperoxalaemia is primarily associated with decreased renal function.

Hyperoxaluria in recurrent non-PH calcium oxalate stone formers

The presence of moderate hyperoxaluria in adult recurrent calcium oxalate stone formers is controversial. In one study, supersaturation with regards to calcium oxalate in the urine of stone formers and non-stone formers was reported to be not significantly different, but the total particle volume in the stone formers were found to be elevated indicating that this group have less inhibitory activity of crystal growth[94]. In other studies, the prevalence of hyperoxaluria in calcium oxalate stone formers has been estimated to be in the range of 10-50%[95-97], with no[95], or a strong[98] correlation between urinary calcium and oxalate.

In children, the frequency of hyperoxaluria in urolithiasis and/or nephrocalcinosis has been reported to be approximately 20% (21 out of 106) [99]. Of the 21 children with increased urinary oxalate excretion found in the above study, eleven had PH (PH1 in nine and neither PH1 or PH2 in two), secondary hyperoxaluria was found in six (two enteric and four dietary) and four could not be classified. In the non-PH patients the colonization of OF in the gut was tested and found absent in all but one.

The reason for the discrepancy in the estimation of hyperoxaluria prevalence in stone formers is not obvious, but differences in methodologies used for oxalate measurements may play a role. In addition, if urinary stones are present at the time of urine collection, the urine sample might be depleted of lithogenic substances as the stones continuously increase in size by incorporating material from urine[100].

As a result, substantially lower concentrations e.g. of oxalate might be determined resulting in false interpretation of urinary risk profile.

The plasma level of oxalate in paediatric calcium stone formers with normal renal function has been reported to be higher (that is, secondary hyperoxalaemia) than in controls, especially in those with increased urinary oxalate, indicating that the intestinal oxalate absorption might be a significant variable influencing plasma oxalate[101].

Epidemiology of kidney stones

Epidemiological studies has revealed that the probability of forming kidney stones (nephrolithiasis) differ in various parts of the world being lowest in Asia, medium in Europe and highest in Saudi Arabia [102]. The chemical composition of urinary stones varies with geographical area, sosioeconomic conditions and climate. The incidence of nephrolithiasis in western countries has been progressively increasing over the past century, which has been attributed to changes in dietary habits and lifestyle [12]. The lifetime risk of nephrolithiasis is about 10-15% in the developed world and throughout adult life is slightly more common in males than in females [49].

Regarding stone composition, calcium oxalate and/or mixed stones are more frequent in young people [103]. Overall, calcium oxalate (alone or in combination) accounts for 60-80% of all urinary stones and is thus by far the most common constituent [48].

Interestingly, kidney stones is known to be more frequent in white subjects than in black subjects but the underlying mechanisms of the racial difference are not clear[102]. Urinary calcium has been reported to be lower in black subjects compared to white subjects on similar diets [104;105]. In addition to the racial difference in urinary calcium, higher pH is found in urine from black women compared to white[106]. By testing the effect of five different dietary and supplemental challenges on urinary risk factors for calcium oxalate stones in comparable groups of healthy white and black subjects, Lewandowski et al[105] found that the white subjects were much more sensitive to dietary changes. Based on these findings they speculated that Blacks apparent immunity to nephrolithiasis are due to a renal or gastrointestinal homeostatic adjustment keeping urinary concentration of lithogenic substances in balance[105].

Factors affecting calcium oxalate nephrolithiasis

A number of factors affect calcium oxalate nephrolithiasis. The role of diet, colonization with OF and the role of calcium will be discussed in the following.

The role of dietary oxalate in the pathogenesis of calcium oxalate nephrolithiasis is not clear[15]. In a prospective study of more than 240 000 adults, food frequency questionnaires were used to asses oxalate intake and the incidence of nephrolithiasis. The mean oxalate intakes were found to be 214 mg/d in men, and 185 and 183 mg/d in older and younger women, respectively. No significant difference in oxalate intake between stone formers and

non-stone formers was observed[107], implying that dietary oxalate is not a major risk factor for nephrolithiasis.

In a different study designed to assess the role of dietary oxalate on hyperoxaluria in calcium oxalate stone patients, 24-h weighed dietary record and 24-h urine from 93 stone formers with, and 93 stone formers without increased urinary oxalate was compared. Interestingly, no significant differences in the amount of dietary oxalate or calcium were found between the groups suggesting that hyperoxaluria in calcium oxalate stone formers at least partly results from intestinal hyperabsorption of oxalate[108].

This hypothesis was further supported by Voss et al[14] who compared the absorption of $^{13}C_2$ oxalic acid in 120 idiopathic calcium oxalate stone formers and 120 controls. They found that the intestinal oxalate absorption was higher in the stone formers than in the healthy controls. Oxalate absorption greater than 10% was found in 45.8% of the stone formers in comparison to 28.3% in healthy volunteers and they suggested that the harmless $^{13}C_2$ oxalic acid absorption test should be routinely used to identify patients with higher oxalate absorption to assist recommendations for individual therapy. Greater oxalate absorption in stone formers than non-stone formers was also reported in other studies [14;37], while one study found no difference in either intestinal absorption or renal handling of oxalate between these groups of individuals [15].

Regarding the role of calcium, based on studies on normal and stone-forming populations there is a general agreement that the mean calcium excretion in stone formers is higher than in the normal population[5;15].

The historical underestimated role of oxalate in determining the risk of forming calcium oxalate stones has been suggested to result from the difficulties in detecting and measuring oxalate reliably in urine. During the same period, the measurement of urinary calcium was performed with reasonable accuracy, and thus patients with calcium containing stones were assessed by hypercalciuria, and decreased intake of calcium was a dietary advice[3].

However, among others, Holmes et al[5] reported a significant decrease in oxalate excretion with increased calcium intake. Recently it was shown that gastrointestinal binding of oxalate by inclusion of calcium-containing foods in meals was an effective clinical strategy for prevention of hyperoxaluria. By increasing the calcium intake in calcium-oxalate stone formers, both oxalate excretion and calcium oxalate supersaturation was found to decrease while urinary calcium excretion remained unchanged[97]. The decrease in urinary oxalate with increased calcium intake was also found by Matsumoto et al in healthy subjects on liberal oxalate intake, but they reported a higher saturation of calcium oxalate following higher

calcium intake as the decrease in urinary oxalate did not overcome the effect of increased calcium[109]. They therefore concluded that a high calcium diet and liberal oxalate intake may pose an increased risk of calcium oxalate stone formation. It seems reasonable to conclude that a combination of mild dietary oxalate restriction in combination with a normal calcium intake would give the best protective effect.

The oxalate to calcium molar ratio in urine is about 1:10, thus an increased intestinal absorption of oxalate may lead to hyperoxaluria that significantly enhances the risk of formation of urinary stones [13]. The reason for the much stronger effect of an increase in urinary oxalate compared to an increase in calcium on the supersaturation of urine is complicated, but basically it can be explained in the following way: As oxalate ($C_2O_4^{2-}$) is present in a much lower concentration than calcium (Ca^{2+}), an increase in oxalate does not significantly reduce the concentration of Ca^{2+} by complexation, and the product $[Ca^{2+}] \times [C_2O_4^{2-}]$ rises almost proportionally to the increase in oxalate concentration. In contrast, an increase in the concentration of ionized calcium is almost entirely offset by a proportional decrease in that of oxalate. As a result, the product of $[Ca^{2+}] \times [C_2O_4^{2-}]$ remains almost constant in the range of normal to elevated urinary calcium[98].

Regarding the role of colonization with OF, Kaufman et al[25] performed a case-control study of 247 adult patients with recurrent calcium oxalate stones and 259 matched control subjects and found that colonization with OF was associated with a 70% reduced risk of being a recurrent calcium oxalate stone former.

Enteric hyperoxaluria

Intestinal overabsorption of oxalate with attendant hyperoxaluria has been reported in several medical conditions with malabsorption. Under normal circumstances calcium binds to most of the intestinal oxalate. Intraluminal free fatty acids can form complexes with calcium, but in malabsorption the concentration of intraluminal free fatty acids is increased and this completely inhibits the precipitation between dietary oxalate and calcium thus leading to more oxalate being available for absorption[110].

Intestinal disease or surgery

After bariatric surgery for weight loss a high prevalence of hyperoxaluria in adult patients without a history of kidney stones has been reported [111;112]. Even non-reversible renal failure

due to oxalate induced nephropathy has been reported [113].The reason for the observed hyperoxaluria in these patients has not been defined, but probably involves malabsorption of fatty acids and bile acids resulting in an increased intestinal absorption of oxalate[114].
As mentioned under ”absorption of oxalate”, non-hereditary elevated urinary oxalate but also acute renal failure with findings of oxalosis on renal biopsy following bariatric surgery has been documented[111;112] and suggests that hyperoxaluria may be a common underlying risk factor for calcium oxalate nephrolithiasis following this surgical procedure[114].
Children with chronic diarrhoea and short bowel syndrome has been shown to be at risk of developing enteric hyperoxaluria due to malabsorption[110]. Cuvelier et al described a patient who received two consecutive renal transplants, both with early graft failure. Biopsies of both grafts revealed widespread oxalate deposition suggesting acute oxalate nephropathy. The diagnosis of chronic pancreatitis (leading to fat malabsorption) causing enteric hyperoxaluria was then made [115]. Renal transplantation as a successful treatment of end stage renal disease secondary to enteric hyperoxaluria in a patient with Chrohn’s disease has also
been described [116].
A possible link between the use of the immunosuppressive drug mycophenolate and acute renal failure in a recipient of a simultaneous kidney-pancreas transplant has been suggested. The patient had prolonged mycophenolate-assosiated diarrhoea and presented with acute renal failure caused by oxalosis. By replacing mycophenolate with a different immunosuppressant (azathioprine), symptoms greatly improved [117]. However, the link between mycophenolate and hyperoxaluria has been questioned by others[118].
It has also been shown that the use of a weight loss drug orlistat (a gastrointestinal lipase inhibitor), decreasing dietary fat absorption, increases oxalate absorption and leads to a significantly increased urinary oxalate levels that again may increase the risk of stone formation. The lipase inhibitor increases the amount of free fatty acids that binds to calcium in the intestinal lumen, hereby decreasing the availability of calcium for binding of oxalate. As a result the concentration of soluble oxalate increases resulting in increased absorption [119]. “Orlistat-induced acute oxalate nephropathy” has recently been described [120].

Pancreatic insufficiency

Several cases of alcohol-related chronic pancreatic insufficiency causing diabetes and steatorrhea presenting with rapidly progressive renal failure has been published. The high

rate of deterioration of renal failure in these patients it thought to be a result of the synergy between enteric hyperoxaluria and a reduction in renal excretory function caused by early diabetic nephropathy[121]. In a case of late renal transplant dysfunction due to acute oxalate nephropathy, a diagnosis of enteric hyperoxaluria secondary to pancreatic insufficiency was made. This had occurred because the patient had been non-compliant with his pancreatic enzyme replacement therapy. After treatment to reduce the amount of circulating oxalate was initiated (haemodialysis, low oxalate and fat diet, appropriate pancreatic enzyme replacement therapy), graft function subsequently recovered[122].

Kidney failure and hyperoxalaemia

Glomerular filtration rate (GFR) describes the flow rate of filtered fluid through the kidney. Adjusted for body surface area, typical GFR values in adults are 100-130 ml/min/1.73M^2. Calculation of GFR can be done by measuring any compound that has a steady level in the blood, and that is freely filtered but neither reabsorbed nor secreted by the kidneys. Creatinine, a break down product of creatine phosphate in the muscle, is freely filterable but actively secreted in only very small amounts. The plasma concentration of creatinine is therefore commonly used as an indirect marker of kidney function. It has been found that in patients with renal failure unrelated to primary hyperoxaluria, the retention of oxalic acid increases rapidly when GFR decreases below about 20 ml/min[123].
When the plasma oxalate is increased formation of calcium oxalate crystals may cause tubular injury. As renal the function decline, the urinary excretion of oxalate falls causing a further increase in plasma oxalate setting up a vicious circle.

Kidney transplantation and hyperoxalaemia

After renal transplantation in end-stage renal patients, accumulated oxalate will be excreted creating an increased risk of tubular precipitation, especially in the presence of allograft dysfunction as indicated in several biopsy studies [124-126].
Controversy exists regarding the degree of oxalosis as a complication of chronic renal failure. By studying and comparing the occurrence of renal and myocardial oxalosis at autopsy in patients with normal renal function, acute renal failure, chronic renal failure, and chronic renal failure supported by haemodialysis or peritoneal dialysis, Salyer et al[127] found

that the incidence and severity of oxalosis was related to the duration of renal failure. Extensive renal and myocardial deposits were frequently found in patients with renal failure of long duration, but in those on haemodialysis (but not on peritoneal dialysis) less oxalosis was found suggesting that haemodialysis ameliorated the oxalosis to some extent[127].

The effect of haemodialysis on oxalosis was further studied by Fayemi et al[128]. The incidence and organ distribution of oxalosis in a group of 80 patients with chronic renal failure maintained on haemodialysis for periods ranging from three weeks to seven years was examined. The most frequently involved organs were the kidneys, thyroid, and myocardium, and moderate to severe renal oxalosis was found more frequently in patients maintained on haemodialysis for longer periods of time[128]. In the above study, bone oxalosis was not studied, but in a patient maintained on haemodialysis for more than four years severe oxalosis of bone was found[129]. Surprisingly, although there was a history of "renal disease" in this patient's brother, it seems that the possibility of primary hyperoxaluria in this patient had not been considered.

To study the magnitude of oxalosis in bone in cases of ESRF, Marangella et al[67]compared the bony content of oxalate on biopsies of the iliac crest in uremic PH-patients and non-PH patients who had been on dialysis treatment for several years. In PH patients, but not in the non-PH patients, the amount of oxalate accumulated in bone was significantly related to time on dialysis. In the non-PH patients, only slightly increased bone oxalate was found compared to controls (0.8, 5.1 and 362 μmol oxalate/g bony tissue in controls, non-PH and PH patients, respectively). In addition, no relationship was found in either group between bony oxalate and pre-dialysis plasma levels. The authors concluded that oxalate deposition in bone was not a progressive disorder in non-PH patients with ESRF[67], in agreement with the earlier findings of Tomson et al[130] (see under). In contrast to the above findings, oxalosis extensively involving bone in patients with chronic renal failure of long duration has been reported [131;132].

6. Plasma oxalate concentration and oxalosis

The first study on the association between plasma oxalate concentration and tissue deposition in non-PH patients was reported in 1988. Plasma oxalate was measured postoperatively in a group of patients undergoing renal transplantation, and in a group of patients with chronic renal failure in whom plasma oxalate had been measured before death. Mild to grossly elevated plasma oxalate was detected in both groups. Renal deposition of oxalate was found in half of the patients and was associated with a plasma oxalate concentration of >20 μmol/L

(upper reference limit with the reported methodology: 1,5 μmol/L). Although many different tissue types were examined (but not bone), no extra-renal deposits were found in any of the patients. The authors concluded that non-PH patients with chronic renal failure is not at great risk of extra-renal oxalosis [130]. In a different study, the same researchers found that the elevated plasma oxalate found in ESRF was not an important risk factor for cardiac disease or vascular calcification in patients maintained on continuous ambulatory peritoneal dialysis[133]. Borland et al[134] compared serum oxalate in patients with chronic renal failure given low-protein diet and treated with chronic haemodialysis, peritoneal dialysis as well as in others not yet under dialysis treatment. They found that serum oxalate did not reach abnormal levels until a very late stage in chronic renal failure [134]. However, they used a method for oxalate measurement involving initial alkalization of serum and reported an upper reference limit of 55μmol/L which seems erroneously high.

For the formation of calcium oxalate crystals to occur, a state of supersaturation with respect to this salt is necessary. It is therefore reasonable to assume that exceeding the solubility limit in plasma represents an actual risk for the deposition of calcium oxalate crystals in body tissues. Theoretically, a selective accumulation of calcium and oxalate within a tissue is an alternative mechanism of the crystal formation. By measuring and comparing the serum supersaturation with respect to calcium oxalate in adult patients who were either uremic or had chronic renal insufficiency not requiring dialysis, and healthy controls, Worcester et al[66] concluded that supersaturation was common in chronic renal failure. The calcium oxalate supersaturation was found to correlate strongly with oxalate concentration, and above oxalate levels of about 40 μmol/L supersaturation occurred. Further, a regression relationship between plasma creatinine (as indicator of the level of renal failure) and plasma oxalate in adults was constructed indicating that a plasma creatinine of 9 mg/dL (795

μmol/L) would almost universally result in supersaturation[66]. A slightly different result was found by Marangella et al[135] who calculated the state of saturation with respect to calcium oxalate monohydrate before and after dialysis in PH patients and non-PH patients on regular dialysis treatment. As expected the plasma oxalate concentrations found were elevated in all patients and fell after dialysis, but in contrast to the samples from the PH-patients, samples from the non-PH patients were only slightly supersaturated before dialysis. These finding indicated that dialysis treatment may maintain body fluids below the risk of calcium oxalate crystallization, unless the underlying disease is PH. In agreement with Worcester et al, the major determinant of the state of saturation with respect to calcium oxalate was found to be the plasma oxalate concentration [135]. The discrepancy between the level of supersaturation reported in the two studies may at least partly be due to differences in analytical methods used and consequently normal range reported for oxalate in plasma, mean normal concentration 3,8 μmol/L in [135] and 10 μmol/L in [66].

In children with chronic renal failure not due to PH, systemic oxalosis is not a common finding, but supersaturation with respect to calcium oxalate has been observed when the renal function is only moderately reduced, and occurs when the plasma oxalate levels reaches about 30 μmol/L (Ion chromatography, mean normal concentration 6,43 μmol/L) [68]. Even in children with ESRD, systemic oxalosis is not a common finding unless the underlying disease is PH. The plasma concentration of crystal inhibitors like citrate and sulphate in children has been reported not to differ between PH and non-PH ESRD-patients but the plasma oxalate concentration is considerably higher in PH suggesting that plasma oxalate measurement alone is sufficient for determining the risk of systemic oxalosis[136]. The reason why plasma calcium oxalate supersaturation does not result in development of oxalosis in non-PH patients with ESRD is not fully understood, but may be due to a combination of the observed return to calcium oxalate undersaturation after dialysis in non-PH patients but not in PH patients and perhaps the presence of unknown, protective factors that prevent the risk of systemic oxalosis in non-PH children with ESRD[136].

The discrepancy between the findings in different studies on renal failure and oxalosis may be partly due to the fact that other factors than the plasma saturation of calciumoxalate may play an important role in oxalosis. It has been showed that the occurrence of prior tissue damage could represent a crucial predisposing factor in the deposition of calcium oxalate crystals[137]. In rats with chronic renal failure, the effect of increased plasma oxalate, elevated plasma ionized calcium and local myocardial tissue damage on myocardial deposition of

calcium oxalate was investigated. Interestingly, neither increased plasma levels of calcium, oxalate, or both, induced myocardial calcium oxalate crystal deposition (but massive renal deposition was found). But, after heterotropic cardiac transplantation (by transplanting into the abdomen a heart allograft), elevated oxalate resulted in significant oxalosis in the heart allograft, but not in their own heart, indicating that the lack of integrity of local tissue may play an important role in oxalosis[137].

7. Renal transplantation in Norway

Oslo University Hospital Rikshospitalet is the only transplant centre in Norway. It is by far the biggest kidney transplant centre in Europe and the number of kidney transplants performed approaches 300 during a year. For patients with ESRF, renal replacement therapy (RRT) by haemodialysis, peritoneal dialysis or kidney transplantation is active treatment options. Transplantation has in general been considered the treatment of choice, after the introduction of the kidney transplant program in Norway in 1969. Best results have been obtained with a living related donor. The acceptance criteria for transplantation have been generous, and in principle transplantation is offered to all patients considered to profit from it. No strict upper or lower age limit has been applied. The results from transplantation of patients beyond 70 years have been surprisingly good during the last decade [138]. Pre-emptive transplantation, that is, transplantation performed before the initiation of chronic maintenance dialysis, has always been preferred since the start of an organized transplantation programme in Norway [139]. For data presented in this thesis, patients were recruited during the years 2004 and 2005. Some key numbers related to renal replacement therapy and kidney transplantation in Norway for the years 2004, 2005, and 2008[140-142] are summarized in Table 2.

Table 2. Key numbers in renal replacement therapy (RRT) and renal transplantation in Norway, 2004, 2005, and 2008.

Variables	**Year**		
	2004	**2005**	**2008**
Patients receiving RRT	3498	3383	4225
% males/females	63.9/36.1	64.5/35.5	67.7/32.3
New patients entering RRT	459	459	533
Patients starting dialysis who were previously unknown to the renal unit	23 %	29%	30%
Death in RRT (% of total in RRT)	254 (7.3)	314 (8.4)	330 (7.8)
Renal transplants at OUH Rikshospitalet	265	229	278
First/second/third/fourth grafts	229/29/7	191/33/4/1	224/48/6
Graft from living donor (%)	95 (35.8)	87 (38)	98 (35)
Pre-emtive transplantation	29 %	33%	43%
Age range, first graft recipients. Years (mean)	2-73 (41.7)	2-75 (44.7)	6-74 (46.7)
Graft from diseased donor	170	142	180
Pre-emtive transplantation	18 %	16%	22%
Age range, first graft recipients. years (mean)	1.5-78 (55.8)	8-80 (56.5)	22-82(55)

8. Determination of oxalic acid in biological samples

Reliable determination of oxalic acid in biological matrices has over the years proven to be very difficult, as evident from the very large number of methods which have been published on this topic only to be discarded and replaced by others.

Methods based on a wide range of analytical principles have been used, and in the 1980'ties and 90'ties several review articles on the different methods available up until then was published [143-147].

Some methods have been based on detection of the native compound (oxalic acid itself), some on determination of the products of a chemical reaction between oxalic acid and other compounds (e.g. H_2O_2 formed by enzymatic conversion of oxalic acid), and some on detection of chemically modified oxalic acid (e.g. di-esters formed by derivatization).

For most of the methods, some sort of sample preparation was needed to remove interfering compounds. For urine, this has generally been achieved by precipitation of oxalate as its calcium salt, by liquid-liquid or solid phase extraction, ion chromatography or adsorption chromatography. For plasma, removal of proteins is a common initial step of the sample preparation procedure either by ultrafiltration or precipitation by acid/heat. The resulting deproteinized plasma could then undergo the same procedure as urine. The concentration of oxalic acid in plasma is considerably lower than in urine and in addition the sample matrix is more complex and thus more extensive sample preparation procedures are often required. A phenomenon known as *in-vitro* oxalogenesis also contributes to the difficulty in obtaining reliable measurement of oxalic acid, especially in plasma. As a result the number of methods published on determination of oxalic acid in plasma is considerably lower compared to methods for urine analysis.

8.1. *In-vitro* oxalogenesis

In-vitro oxalogenesis, or simply oxalogenesis, is a process in which compounds present in the sample is converted to oxalic acid after sample collection and/or during assay. The result of oxalogenesis is erroneously elevated oxalic acid measured. As early as in 1933 it was reported that ascorbic acid could be oxidized to oxalic acid [148], but this fact was in the following decades apparently forgotten by the analytical chemists developing methods for oxalic acid determination. The instability of oxalic acid in biological fluids was observed, but the identity of the precursor(s) of oxalic acid responsible for oxalogenesis was for a long

time not known. However, it was known that glycolic acid could be oxidized to oxalic acid and in 1980 Akcay et al published data suggesting that glycolic acid was the source of oxalogenesis.
Several researchers tried, but failed to reproduce these findings [149;150]. The fact that ascorbic acid (vitamin C) could be oxidized into oxalic acid was rediscovered in the mid 1980'ties[151], and since then it has been generally accepted that ascorbic acid is the main source of in vitro oxalogenesis[152;153]. The breakdown of ascorbic acid into oxalic acid (and threonic acid) goes through several steps in which the last is highly pH dependent and only taking place at alkaline pH. A number of different sample preparation procedures including removal of ascorbic acid or acidification of sample have been suggested and adapted to prevent oxalogenesis.

An overview of the different sample preparation procedures and analytical systems reported up to date for oxalic acid determination in plasma and urine follows.

8.2. Sample preparation

The sample preparation procedures used in methods for oxalic acid determination differs widely depending on the proceeding analysis technique (e.g. chemical analysis or gas chromatography). Therefore, details on the sample preparation procedures adapted are in the following given together with the analytical procedure described.
However, a brief description on the three most commonly used sample preparation principles for oxalic acid determination, calcium oxalate precipitation, liquid-liquid extraction and solid-phase extraction, will first be presented.

Calcium oxalate precipitation

The precipitation of oxalic acid at alkaline pH as its calcium salt was by far the most commonly used cleanup process for the earlier methods of oxalic acid determination. The solubility of calcium oxalate is strongly pH dependent being practically insoluble at alkaline and neutral pH but soluble at low pH. The main drawback of calcium oxalate precipitation is the very long time needed for quantitative precipitation, the co-precipitation of other salts such as calciumphosphate, incomplete precipitation, and losses of calcium oxalate during washing[145].

Liquid-liquid extraction and solid-phase extraction

Extraction procedures are typically used as part of a sample preparation procedure to reduce the complexity of a biological sample prior to further analysis.
Liquid-liquid extraction is a sample cleanup process that is based on separation of compounds according to their relative solubility in two immiscible liquids, usually water and an organic solvent. In the earlier methods for oxalic acid analysis especially in urine, liquid-liquid extraction was widely used. The fact that oxalic acid is very water soluble makes it difficult to extract into organic solvents, and thus very long extraction times or repeated extraction had to be done to obtain acceptable recoveries.

Solid phase extraction (SPE) is a sample cleanup process that is used to remove compounds from a mixture by using the chemical and physical properties of the analyte.
SPE is typically used to purify and concentrate the analyte before further analysis. The principle of SPE is liquid chromatography (see later); it is based on the affinity of the analyte dissolved in a liquid (mobile phase) for a solid (stationary phase) through which the sample is passed.
A variety of stationary phases are available for SPE. Most of them are based on silica bonded to a specific functional group such as quarternary ammonium groups for anion exchange SPE and hydrocarbon chains of variable length like octadecyl or octyl carbon chain (C18 and C8, respectively) for reversed phase SPE.

Depending on the choice of SPE conditions, either the analyte of interest or the undesired impurities can be retained on the SPE material. For oxalic acid, the use of anion exchange SPE is an example of setup where the analyte is retained on the stationary phase, while the use of a C18 resin will allow the oxalic acid to pass through the material unretained while neutral and hydrophobic components in the sample are retained.

If the analyte is retained on the stationary phase, a washing step is typically subsequently performed to remove unretained and weakly retained compounds. Finally an elution step is performed in which a solution that disrupts the interaction between the analyte and stationary phase is used to elute the analyte in a small volume.

8.3. Analytical methods for determination of oxalic acid

In the following an overview, including discussion on main advantages and disadvantages, of the majority of methods that has been developed for oxalic acid determination up until today is given.

8.3.1. Chemical techniques

Oxalic acid has been measured directly by quantification of it s oxidation products, or after initial reduction to glyoxylic acid or glycolic acid, and this was the basis of many of the early methods described.

Oxidation in combination with titrimetry or manometry

Oxalic acid can be oxidised by acidified potassium permanganate:

$$5H_2C_2O_4 + 2KMnO_4 + 3H_2SO_4 = K_2SO_4 + 2MnSO_4 + 8H_20 + 10CO_2$$

This reaction has been the basis for many of the early methods for oxalate determination after initial precipitation of oxalate as calcium oxalate. As an alternative to precipitation of calcium oxalate, the precipitation of oxalate from serum as its cerium salt has also been suggested [154]. However, this procedure was criticized by several authors and the existence of oxalate in the precipitate questioned [155].

In the titrimetric methods, typically the persistence of the pink colour of potassium permanganate was used to determine the end-point of the titration. For analysis of oxalate in urine, an initial sample cleanup by liquid-liquid extraction of the acid into boiling peroxide-free ether before precipitation of calcium oxalate was suggested to remove interfering compounds in the sample. Satisfactory recovery was obtained only after 18 hours of extraction [156].

For serum, manometric determination of the CO_2 evolved in the permanganate reaction has also been used to quantify oxalate. After an initial acidic precipitation and removal of plasma proteins, calcium oxalate was precipitated, supernate removed and precipitate redissolved with sulphuric acid before addition of permanganate and manometric determination of the CO_2 produced[157]. An alternative sample preparation procedure for the

oxidation-manometry method was later described in which oxalate was quantitatively extracted from serum by esterification and distillation [158].

In general, the methods based on precipitation of oxalate followed by oxidation by permanganate were very time consuming and great care had to be taken to standardize the temperature of the reaction. The fact that permanganate reacts with many other reducing substances especially in urine resulted in a general underestimation of oxalate concentration in methods based on the permanganate reaction.

Reduction in combination with fluorimetry or colorimetry

Oxalic acid can be reduced to glyoxylic acid in the presence of zinc and hydrochloric acid:

$HO\text{-}CO\text{-}CO\text{-}OH + H_2 = H\text{-}CO\text{-}CO\text{-}OH + H_2O$

A fluorimetric method that initially was developed for quantification of glyoxylic acid[159], was adapted for determination of oxalate in serum and urine. Proteins from serum or urine were removed by acid/heat precipitation. Before calcium oxalate precipitation, the samples were processed by liquid-liquid extraction with tri-n-butyl phosphate as this solvent was found to have a much higher partition coefficient for oxalate than e.g. diethyl ether. Oxalic acid was then reduced in the presence of zink and hydrochloric acid. The resulting glyoxylic acid was allowed to react with resorcinol, forming a fluorescent glyoxylic acid-resorcinol complex that had maximum emission at 530 nm[160].

The conditions used for the reduction of oxalate to glyoxylate was critical as the reaction was difficult to control at the glyoxylate stage, and most workers therefore preferred to utilize the reduction of oxalic acid into glycolic acid :

$HO\text{-}CO\text{-}CO\text{-}OH + 2H_2 = HO\text{-}CH_2\text{-}CO\text{-}OH + H_2O$

The glycolic acid produced could readily be detected with colorimetry. By heating glycolic acid with concentrated sulphuric acid and chromotropic acid, the formaldehyde formed from breakdown of glycolic acid formed a purple complex with chromotropic acid and this complex was detected at 570 nm. In 1961 Hodgkinson and Zarembski described a method for determination of oxalate in urine using this principle. Continuous extraction of acidified urine with peroxide-free boiling ether for 6 hours was performed. The extracted oxalic acid was then precipitated as the calcium salt, reduced to glycolic acid by boiling with zinc and sulphuric acid and determined colorimetrically after addition of chromotropic acid[156]. However, the initial heating of acidified urine was thought to cause positive interference by converting oxaluric acid present in urine to oxalic acid. Ten years later an improved method

for oxalate in urine was published by the same research group, based on two previous procedures ([156] and [160]). Solvent extraction was omitted and the oxalic acid was co-precipitated directly from urine with calcium sulphate and ethanol. The precipitated oxalic acid was then converted to glycolic acid and determined colorimetrically as before [161]. Glucose in the concentration found in diabetic urine was found to cause significant interference. The same did glycolic acid present in concentrations found in primary hyperoxaluria.

Using this method, normal men were found to excrete from 17-43 mg oxalic acid per day. The reduction/colorimetric procedure have also been used for determination of oxalic acid in plasma. Instead of liquid-liquid extraction, solid phase extraction using an ion-exchange resin was used for removal of interfering substances from plasma [162]. In general, the chemical methods involved time consuming sample preparation based on precipitation or continuous liquid-liquid extraction and also the use of strong acids. Since the end of the 70'ties the chemical methods for determination of oxalic acid was largely replaced by enzymatic methods and also by methods based on chromatography and mass spectrometry.

8.3.2. Isotope dilution techniques

Isotope dilution techniques are analytical techniques that involve the addition to a sample of an isotopically labeled compound, either stable or radioactive. Long before isotopes were discovered, zoologists started using catch and release methods for estimation of fish populations in ponds. The principle behind this was very simple: a certain number of fish was caught, labeled, and released back into the pond. After the released fish was intermingled with all other fish in the pond, a second catch was performed and the ratio of labeled and unlabeled fish recorded. Since the total number of labeled fish was known, this ratio allowed estimation of the total fish population in the pond [163]. As an example, with five labeled fishes and a ratio of labeled to unlabeled fish in the second catch being 1: 4, an estimated 25 fish was in the pond. In isotope dilution, a known amount of labeled fish would be added to the pond and then the ratio of labeled-to-unlabeled measured from a representative sample, and this demonstrates the working principle of isotope dilution.

For determination of oxalic acid, both isotope dilution with the radioactive isotope ^{14}C and the stable isotope ^{13}C has been used. In early methods for determination of oxalic acid in urine by isotope dilution, $^{14}C_2$ oxalic acid was added to urine followed by overnight precipitation as calcium oxalate. The precipitate was washed, dried, and dissolved in

sulfuric acid with added zinc for reduction of oxalic acid to glycolic acid. After separating glycolic acid from other compounds on an ion-exchange column, aliquots were taken for ^{14}C counting in a scintillation counter, and for colorimetric determination of glycolic acid[164]. The content of oxalic acid in urine was then calculated from the specific activity of the isolated glycolic acid (derived from oxalic acid) and the total counts of $^{14}C_2$ oxalic acid added to the specimen (total counts of $^{14}C_2$ oxalic acid – specific activity of derived ^{14}C glycolic acid = oxalate content of the specimen). Using this technique, the daily urinary excretion of oxalate pr 1.73 m^2 of body surface in healthy children averaged 32.9mg, in children with PH 149-375 mg was found, and in healthy adults 18-47 mg.

The concentration of oxalic acid in plasma is considerably lower than in urine, and in general far more methods have been described for oxalate urine analysis compared to plasma. At the same time methods for direct determination of oxalate in urine was readily available, but direct determination of oxalic acid in plasma was difficult and this led to the development of methods for indirect estimation of oxalic acid in plasma by use of isotope dilution. In this indirect method, radioactive oxalate, $^{14}C_2$ oxalic acid, was administrated to the patient himself. Afterwards blood and urine samples were collected. In plasma, only radioactivity was measured, while in urine both radioactivity and oxalate concentration was measured by e.g. colorimetry [165]. The concentration of oxalic acid in plasma was then estimated as: ($^{14}C_2$ oxalic acid activity pr unit volume of plasma) x (concentration of oxalic acid in urine)/ ($^{14}C_2$ oxalic acid activity pr unit volume of urine).

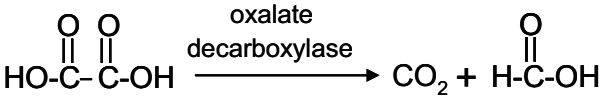
Normal plasma concentrations in the range 0.5 – 1.9 μmol/L was typically found using this technique [166], and 9-19 μmol/L in PH patients[166]. The plasma concentrations estimated using $^{14}C_2$ oxalic acid isotope dilution was and still is of the lowest reported using any technique.

8.3.3. Enzymatic techniques

Enzymes have the advantage of specificity when used as analytical tools in the laboratory. Two enzymes have been used for quantitation of oxalate; oxalate oxidase and oxalate decarboxylase.

Oxalate decarboxylase

Oxalate decarboxylase (EC 4.1.1.2) catalyzes the reaction:



Oxalate decarboxylase can be purified from the wood-rot fungus *Collybia velutipes* and both products of the enzymatic reaction have been used for quantitation of oxalate as outlined in Figure 5.

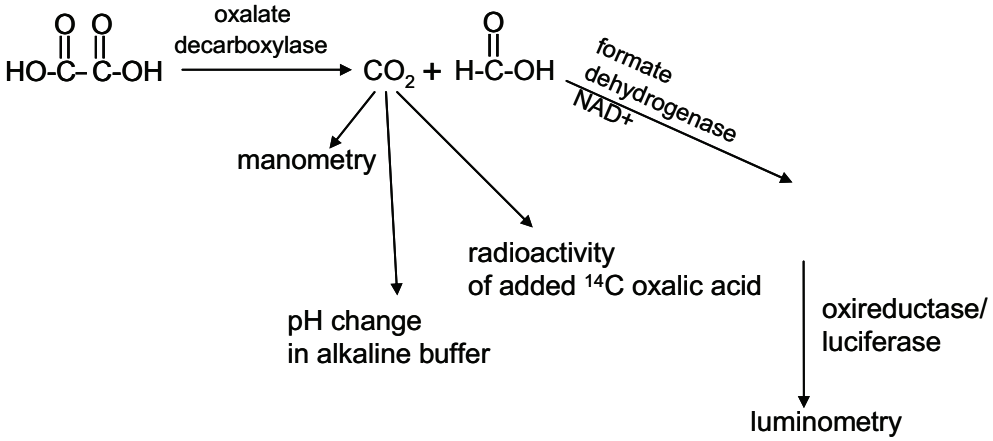


Figure 5. Different approaches for determination of oxalic acid by use of oxalate decarboxylase.

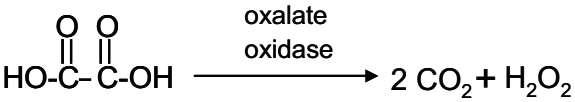
Initially oxalate decarboxylase was used for quantitation of oxalate in plasma, and the CO_2 produced was measured by Warburg manometry [167]. A few years later the same manometric procedure was used for urine analysis after initial precipitation of oxalate as calcium oxalate that was redissolved in potassium citrate buffer [168]. Phosphate and sulphate which are normal constituents of urine are inhibitors of oxalate decarboxylase, but by using

a higher concentration of the enzyme this limitation could be overcome thus making initial sample preparation unnecessary. The CO_2 produced was released into an alkaline buffer and the pH change measured[169]. A modified version of this method was also adapted for plasma[170]. A radioenzymatic assay using oxalate decarboxylase has also been described for plasma oxalate. ^{14}C labelled oxalate was added to plasma and the sample was ultrafiltered, calcium oxalate was then precipitated and redissolved followed by liquid-liquid extraction using diethylether, evaporation and redissolving in a citrate buffer. Oxalate decarboxylase was then added and the progress of the enzymatic reaction followed by measuring the $^{14}CO_2$ evolved in a ^{14}C labelled oxalate standard with and without the addition of the extract from plasma containing unlabelled oxalate[171]. The radioenzymatic assay was further optimized by performing a direct rapid precipitation of calcium oxalate[172]. For urine analysis, the preparation of an enzyme electrode with oxalate decarboxylase entrapped in polyacrylamide gel retained over a CO_2 sensor has been reported [173].

Quantitative methods for oxalate based on measurement of the formate produced by oxalate decarboxylase have also been described. The formate produced can be measured spectrophotometrically by using nicotinamide adenine dinucleotide (NAD+)-requiring formate dehydrogenase, and this double enzyme system has been used for both serum and urine analysis[174;175]. For plasma analysis, even a triple enzyme system has been described in which the NADH produced by formate dehydrogenase is determined by a bacterial oxidoreductase/luciferase system emmiting light that can be quantified in a luminometer [176]. Plasma was treated with a cation exchange resin to adjust pH and remove cations before analysis. An immobilized version of the triple enzyme system has also been described[177].

Oxalate oxidase

Oxalate oxidase (EC 1.2.3.4) catalyzes the reaction:



The enzyme can be purified from many different sources such as barley roots, several species of moss, and from grain sorghum leaves. As shown in Figure 6, analytical methods have been developed based on the quantitation of both of the products formed.

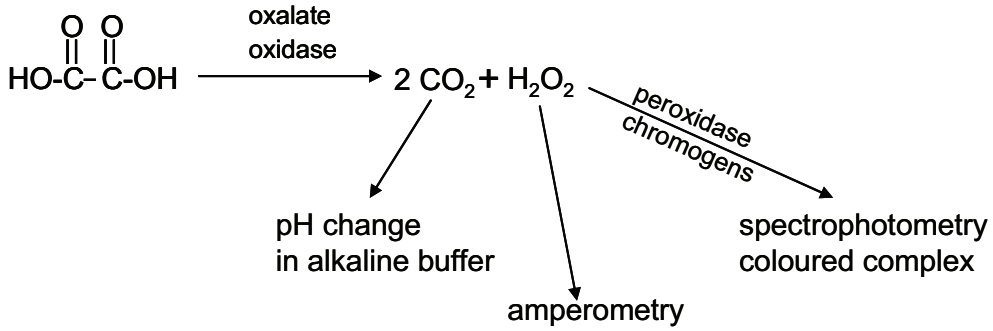
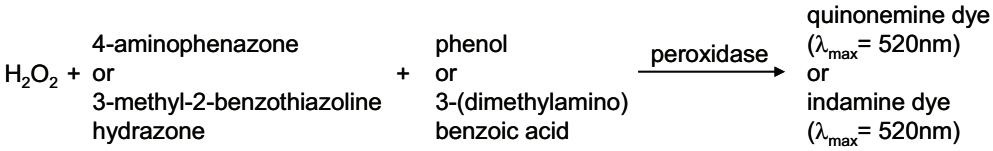


Figure 6. Different approaches for determination of oxalic acid by use of oxalate oxidase.

As for oxalate decarboxylase, the change in pH of a weak alkaline buffer arising from trapping of the CO_2 evolved has been used for quantitation of oxalate, but the oxalate oxidase reaction has the advantage of producing two moles of CO_2 for each mole of oxalate. After deproteinizing by addition of hydrochloric acid and heating, plasma oxalate has been quantified by this technique[178].

A number of methods have been described based on measurement of the H_2O_2 produced by the oxalate oxidase reaction. The use of a second enzyme, horseradish peroxidase that converts H_2O_2 to O_2 and H_2O has been the basis of several methods for oxalate measurement. By mixing the peroxidase with chromogens the O_2 released causes oxidative coupling of the chromogens resulting in the formation of a coloured complex that can be measured spectrophotometrically at 520 or 590 nm:



Oxalate oxidase is inhibited by both divalent metals and several sodium salts including chloride, phosphate and citrate and thus different sample preparation procedures has been described to overcome this limitation. In addition to the potential conversion of ascorbate to oxalate during assay and storage, ascorbate interferes directly with the peroxidase step and needs to be removed prior to analysis. The divalent cations can be removed by an ion exchange resin and ascorbic acid can be eliminated by charcoal treatment, addition of sodium nitrite that converts ascorbate to dehydroascorbate or the use of ascorbate oxidase.
For urine analysis of oxalate, a spectrophotometric method using the oxalate oxidase/peroxidase/coloured dye system in combination with an ion exchange and a charcoal cleanup step was described in 1980[179]. A few years later an immobilized version of this system that allowed reuse was reported. The sample preparation procedure included the initial precipitation of oxalate as calcium oxalate as the authors claimed that alternative methods for removal of interfering components like charcoal and ion exchange were not sufficient[180]. A similar immobilized enzyme system was later developed for plasma analysis in which plasma was first ultrafiltered, acidified and then had sodium nitrite added for ascorbate
elimination [181]. A method suitable for the spectrophotometric determination of oxalate in different biological matrices using the oxidase/peroxidase/coloured dye system was then described in1988. Both plasma and urine could be analyzed, however the sample treatment used was very different including ultrafiltration and precipitation of calcium oxalate for plasma analysis and dilution and pre-treatment with a reversed phase cartridge for urine. The authors found that the use of sodium nitrite interfered with the colour development and the use of this reagent for ascorbate removal was therefore omitted [182]. An immobilized version of this method was later described in which the enzyme was placed in an enzyme coil as part of a continuous flow system. For both urine and ultrafiltered plasma, sodium nitrite was added just before assay [183].
For urine analysis a kit was introduced in 1989 employing an initial alkalization and addition of ethylenediaminetetraacetic acid (EDTA) (avoiding precipitation of calcium oxalate) in combination with charcoal treatment[184]. The kit reagents were later used for plasma analysis. Plasma was ultrafiltered into acid to avoid oxalogenesis and was further treated with ascorbate oxidase for elimination of ascorbate that otherwise caused negative interference probably due to H_2O_2 being reduced by ascorbate [185].
For plasma analysis the removal of proteins by use of sulfosalicylic acid has also been used followed by charcoal treatment of the supernate before assay [186]. The use of oxalate oxidase

purified from sorghum leaves has also been reported. The main benefit of using this source of the enzyme was its insensitivity to chloride present in the sample. Plasma could be analyzed after ultrafiltering into acid and addition of sodium nitrite [187]. Analytical systems based on the immobilization of both oxalate oxidase and peroxidase on glass beads that could be reused has been described for plasma analysis [188]. By affixing the glass beads on a plastic strip the method was further improved [189].

In addition to the number of methods based on the peroxidase-mediated colorimetric procedure, the use of amperometric detection of the H_2O_2 produced has been reported by several investigators. In-line systems with immobilized enzyme (immobilized enzyme reactors, IMER) combined with amperometric detection was introduced for plasma and urine analysis in the 1990'ties. Both ion-pair chromatography and anion-exchange chromatography, respectively, have been combined in-line with the IMER/amperometric H_2O_2 detection [190;191]. For the ion-pair chromatography procedure both plasma and urine was purified using a C18-cartridge after plasma was deproteinized by acid and heat [190]. For the anion-exchange chromatography procedure the C18 cleanup was not necessary, and plasma was analyzed only after ultrafiltering into acid [191;192] and for urine thymol and isopropanol was added for preservation[191]. "Amperometric biosensors" for urine analysis has also been described in which the oxalate oxidase has been immobilized onto an electrode. Samples had to be diluted many times to overcome the need for removing reductive agents present in urine. The H_2O_2 produced reacted with hexacyanoferrate (II) in the buffer solution of a flow-injection system to yield hexacyanoferrate (III) that was reduced back to hexacyanoferrate (II) at an gold electrode[193], or the oxalate oxidase was immobilized on the surface of a chromium hexacyanoferrate-modified graphite electrode[194]. SIRE technology (sensors based on injection of the recognition element) has also been used for urine analysis of oxalate, but the technique was not sensitive enough for plasma analysis. In the SIRE biosensor, the oxalate oxidase was injected into a reaction chamber containing an integrated electrode. A small amount of enzyme was thus used for one measurement and then discarded which greatly increased the lifetime of the biosensor [195].

In summary, out of the many methods published on the use of oxalate oxidase and oxalate decarboxylase, only a few has proven useful for analysis of both plasma and urine. The reported methods are in general labour intensive and special precautions must be made to avoid interference by endogenous substances present in biological samples. The integrated methods with in-line reactors or electrodes involve either the use of special custom made devises and/or the use of enzyme that has been purified in the lab from harvested plants.

8.3.4 Chromatographic techniques

Chromatography is a collective term for a set of techniques used for separation of mixtures. A mixture dissolved in a mobile phase is passed through a stationary phase, and different degree of interaction between the different compounds in the sample and the stationary phase leads to separation of the compounds. In liquid chromatography the mobile phase is a liquid, and in gas chromatography the mobile phase is a gas.

Both liquid chromatography and gas chromatography in combination with several different detection principles has been developed for determination of oxalic acid in biological fluids.

Gas chromatography

Oxalic acid itself is not suitable for gas chromatography (GC) and therefore needs to be chemically modified (derivatized) into a less polar, thermally stable and more volatile compound as part of the sample preparation procedure.
Several methods for oxalic acid determination combining GC and flame ionization detection (FID) were described in the 1970'ties. For GC-FID of oxalic acid in urine, a solid phase extraction procedure with an anion –exchange resin was described for sample clean up. A recovery of 95% of oxalic acid from the anion-extraction resin was found using a radioactive tracer. After elution from the resin, preparation of o-ethyl oxime derivates (to stabilize the sample) was performed before freeze drying over night. The acids in the dried sample were then derivatized into trimethylsilyl esters that was separated and detected by GC-FID[196]. A similar procedure was adapted for plasma analysis, but the acids were derivatized into dimethyl esters before GC-FID analysis[196].
For urine, a GC-FID method without the initial extraction was also described in which 30mL of urine was evaporated to dryness under reduced pressure with a rotating vacuum evaporator followed by ethylation by ethanol/sulphuric acid and GC-FID analysis[197]. A combined method for plasma and urine was also published involving initial evaporation to dryness, liquid-liquid extraction with ether and overnight derivatization with isopropanol and hydrochloric acid followed by GC-FID. The normal concentration found in plasma was 8.9-41 μmol/L and in urine 100-488 μmol/24 h (detection limit 7.5 and 20 μmol/L in plasma and urine, respectively)[198]. A modified version of this urine and plasma method using capillary GC column and without the initial evaporation to dryness was also published.

Ethyl acetate was used for direct repeated liquid-liquid extraction of oxalic acid from acidified urine or plasma. Plasma proteins were found to precipitate in the conditions used during extraction and repeated extraction with the precipitate in place was needed to obtain reliable results. In healthy volunteers, a mean urinary oxalic acid extraction of 230 μmol/24 h was found and plasma concentration in the range 1.3-5.3 μmol/L[150]. GC in combination with electron capture detection has also been used for determination of a chlorinated derivative of oxalic acid in urine after precipitation of calcium oxalate[199].
In most of the GC methods mentioned so far, endogenous compounds present at low concentrations in body fluids, such as malonic acid, was used as internal standards for quantification of oxalic acid. Difference in extraction efficiency and reactivity towards derivatization reagents was an obvious drawback of this approach. The use of GC in combination with mass spectrometry allowed the use of isotopically labeled internal standard that greatly increased the selectivity (See gas chromatography-mass spectrometry below).

Liquid chromatography

In all liquid chromatography (LC) methods the mobile phase is a liquid, but a number of different stationary phases can be used. LC-techniques can be classified according to the polarity of the stationary and mobile phase. In reverse phase chromatography the mobile phase is more polar than the stationary phase, and opposite in normal phase chromatography where the stationary phase is more polar than the mobile phase.
For reversed phase chromatography, stationary phases with hydrocarbon chains of different lengths like C18 or C8 are typically used. The mobile phase contains a mixture of water or aqueous buffers and organic solvents that are miscible with water, like methanol or acetonitrile.
Relatively few reports on the use of reversed phase chromatography for oxalic acid are published. The retention of the highly polar oxalic acid is low on a reverse phase system, but in some reported methods for oxalic acid this was overcome by adding quarternary ammonium ions to the mobile phase as counter ions, hence increasing the retention of oxalic acid[200]. This reversed phase ion-pair LC system with a low pH mobile phase in combination with ultraviolet (UV) detection at 210-220 nm has been used for oxalic acid measurement in urine. Interfering substances had to be removed either using a C18-cartridge[201] or by protein precipitation[202] before analysis on a C8 or C18 column, respectively. The urinary oxalic acid

excretion found in healthy controls was in the range 100 – 225 μmol/24h [202]. Although suitable for urine analysis, poor detection limit (15 μmol/L) did not allow determination of oxalic acid in plasma. Methods based on derivatization of urinary oxalic acid into better retained, more UV-absorbing compounds have also been proposed. By reacting acidified urine with o-phenylenediamine, a highly UV-absorbing oxalic acid derivative was formed that could be detected at 314 nm after chromatography on a C8 column. However, a large background signal was present of unknown origin and thus two separate determinations of each sample had to be performed; one after the urine hade been treated with oxalate oxidase (to remove oxalic acid from the sample) and one without enzyme treatment. The difference in peak area between the two determinations was used for calculation of oxalic acid concentration in urine [203]. A different derivatization approach was also reported using liquid-liquid extraction of urine by tri-n-butyl phosphate followed by derivatization with 9-anthryldiazomethane and C18 chromatography. The resulting fluorescent compound was measured at 410nm with excitation at 254 nm. The derivatized oxalic acid decomposed upon standing and the analysis therefore had to be done within 10h after preparation. [204].
Ion-exchange chromatography, in which the stationary phase is charged, is used for separation of polar or charged molecules. The stationary phase surface displays ionic functional groups that interact with analyte ions of opposite charge leading to retaining of the analyte. Mobile phases typically consists of species that are similarly charged as the analyte and that will displace the analyte ions from the stationary phase.
In combination with conductivity detection, this LC-technique has been the basis for several methods developed for determination of oxalic acid.
In general, the chromatographic column was packed with anion-exchange material and the mobile phase typically consisted of a carbonate/bicarbonate buffer with boric acid. Initially this technique was used for oxalic acid in urine, after only dilution with water and filtering[205]. Sulphate present in urine eluted close to oxalic acid and sulphate therefore had to be added to the aqueous standards used for preparation of the calibration curve in order to overcome the potential problem of peak overlap. For plasma analysis, sample preparation typically involved ultrafiltering to remove proteins that otherwise results in clogging of the chromatographic column. However, it was shown that the time of acidification (before or after ultrafiltering) and the molecular weight cut-off of the ultrafilter used were of significant importance. Sample loss close to 50 % was observed when ultrafiltering was performed with a 30.000 dalton cut -off filter on plasma acidified to pH 3 due to binding of oxalic acid to plasma proteins at this pH. At physiological pH or at extremely low pH,

protein binding was not seen. At the same time oxalogenesis was observed during ultrafiltering if plasma was not first acidified, and initial strong acidification was therefore adapted to obtain satisfactory results[206].

The very pH dependent recovery and the need to acidify to avoid oxalogenesis was a drawback of the method. However, others claimed that plasma did not have to be acidified prior to ultrafiltering, and alternative sample preparation was then proposed in which unacidified plasma was ultrafiltered into acid[207], or into a cation-exchange resin for reduction of pH to about 1[208]. Chloride present in urine could cause interference in the chromatogram and was removed by passing the acidified plasma ultrafiltrate through a silver cation-exchange resin before chromatography. The method was also used for urine, after passing the sample through a C18 cartridge for clean up. In healthy controls, urine excretion in the range 107-560 μmol/day was found and plasma levels in the range 0.8 – 3.2 μmol/L[208]. A simpler sample preparation procedure for plasma oxalate was also described in which proteins were removed from plasma by precipitation with acetonitrile[209]. For work up of unacidified plasma the use of a 10 000 dalton cut-off filter has also been suggested as it reflects the sieve micro architecture of renal glomeruli. Using this procedure, observations suggesting binding of oxalic acid to plasma proteins with a molecular weight higher than the cut off limit was suggested[210], but this was later reported to be apparent and not real by the same researchers[211].

Regardless of the choice of sample purification, a major concern using the ion-chromatographic – conductivity detection methods for oxalic acid is oxalogenesis occurring during chromatography, in the column, from ascorbic acid present in the sample. Ascorbic acid therefore has to be removed before chromatography by e.g. enzymatic oxidation or addition of boric acid to the mobile phase [206].

Although controversy exists on both the impact of in-column oxalogenesis, strategies to prevent it, and choice of sample pretreatment procedure, ion chromatography with conductivity detection is one of the most used analytical systems for determination of oxalic acid in use today.

As an alternative to a charged stationary phase used in ion-exchange chromatography, a custom made solvent-generated ion-exchange chromatographic system with amperometric detection at a copper electrode has been described for measurement of oxalic acid in urine[212].

Capillary electrophoresis

In addition to the number of LC-methods, a CE method using indirect UV-detection has also been reported for analysis of oxalic acid in urine [213]. Although simple and robust, the relative poor sensitivity of CE limited its use to urine analysis.

8.3.5 Mass spectrometry

The principle of mass spectrometry (MS) consists of ionizing a chemical compound in gas phase to generate charged molecules or molecule fragments and subsequent measurement of their mass-to-charge ratio (m/z). A number of different ionization techniques are available like electron impact ionization typically used with GC, or the softer, electrospray ionization (ESI), typically used with LC.

The power of MS lies in the fact that the mass spectra of many compounds are sufficiently specific to allow their identification with a high degree of confidence. If the analyte is part of a complex mixture such as urine or plasma, however, all compounds present in the sample will produce ions and the mass spectrum obtained will be complex. If in addition the analyte is a minor component of the sample, identification with an acceptable degree of certainty is difficult.

The combination of the capability of chromatography to separate compounds in a mixture allowing pure compounds to be introduced into the MS, with the identification capability of MS is clearly advantageous.

After the initial use of isotope dilution with the radioactive isotope, a number of methods based on the use of the stable isotope of oxalic acid, $^{13}C_2$ oxalic acid (1,2-$^{13}C_2$ oxalic acid) was described. Typically, $^{13}C_2$ oxalic acid was added to the sample to work as an internal standard as the labeled analog behaved like the natural compound throughout the analytical procedure hereby correcting for any sample loss. The difference in molecular mass between the ^{12}C and ^{13}C -oxalic acid makes them easy to detect individually in mass spectrometry. The internal standard, 1,2-$^{13}C_2$oxalic acid, has a molecular weigh 2 atomic mass units (u) higher than the natural compound (92.03 and 90.03 g/mol, respectively).

Interfaces for connection of GC with MS was commercially available much before interfaces for connection of LC and MS, and commercially available ESI interfaces has only been available during the last 20 years [214]. The reason for the much earlier routine use of GC-MS is the compatibilities of the two techniques.

Compounds analyzed with GC have to be both volatile at the temperatures needed to achieve separation, and thermally stable. These are by far the same requirements needed to produce mass spectra from an analyte in the MS. Thus, the vast majority of compounds amendable to GC separation can efficiently be transferred to the MS.
This is not the case when LC is interfaced with MS, due to the incompatibilities of the two techniques. Thus, an interface must be used with its prime purpose being the removal of the mobile phase.
After the analyte has been ionized, the ions of different m/z ratios produced are separated and detected. Several different mass separation devices are used in MS. A triple quadrupole mass analyzer is commonly used in combination with LC and ESI.

Gas chromatography-mass spectrometry

Several methods based on gas chromatography-mass spectrometry (GC-MS) using $^{13}C_2$ oxalic acid have been described. In a GC-MS method for urine analysis published in 1979, the $^{13}C_2$ oxalic acid internal standard was added followed by ethanol and $CaSO_4$. After overnight precipitation and drying of calcium oxalate, derivatization reagent was added to the residue followed by heating. The resulting di-propyl ester of oxalic acid was then extracted into hexane and injected on the GC-MS. Oxalic acid eluted after less than 3 minutes from the GC and the protonated molecule formed in the MS after chemical ionization was at m/z 175.0 (endogenous oxalic acid) and 177.0 ($^{13}C_2$ oxalic acid).
Using selective ion monitoring, the ratio of the m/z 177.0 and 175.0 was used to quantify oxalic acid in the urine sample[215]. Several similar methods but with different derivatization procedures was later reported for urine analysis, e.g. with the formation of the di-isopropyl ester of oxalic acid that could be quantified at m/z 194 and 192 (ammonium-adduct of the molecule) for the internal standard and endogenous compound, respectively[216]. As an alternative to the precipitation of calcium oxalate, liquid-liquid extraction of urine with ethyl acetate has been reported followed by lyophilizing and an overnight derivatization procedure resulting in the formation of bis-(tert.-butyl-dimethylsilyl) derivates of oxalic acid that could be quantified by GC-MS with electron-impact ionization. The latter method was developed to monitor intestinal absorption of oxalic acid by administrating $^{13}C_2$ oxalic acid and isotopically labeled malonic acid was actually used as internal standard for both labeled

and unlabeled oxalic acid. The use of ethyl acetate for extraction was not very efficient, and only about 50% of the oxalic acid was retained in the ethyl acetate phase after washing. A mean excretion of 0.335 mmol oxalic acid/24h was found in healthy female volunteers, and absorption of ingested $^{13}C_2$ oxalic acid in the range 1-48% [217]. Stable isotope-dilution GC-MS determination of oxalic acid in plasma has also been described. After initial addition of the isotopically labeled internal standard, plasma was acidified followed by solvent extraction with ethyl acetate and derivatized into trimethylsilyl-derivates that was quantified in the MS[218].

Tandem mass spectrometry

Tandem mass spectrometry (MSMS) is a term that covers techniques in which one stage of MS is used to isolate an ion of interest and second stage of MS is then used to probe the relationship between this ion with others from which it have been generated or generate on decomposition.

The triple quadrupole is probably the most widely used MSMS-instrument. As the name suggests, the hardware consists of three sets of quadrupole rods in series. The second set of rods is not used as a mass separation device but as a collision cell where fragmentation of ions transmitted by the first set of quadrupole is carried out.

The most used MSMS experiments in LC-MSMS are *product ion scan*, *precursor ion scan*, *constant neutral loss scan,* and selected decomposition monitoring or *multiple reaction monitoring* (MRM).

In MRM, the fragmentation of a selected precursor ion to a selected product ion is monitored.

This is performed by setting the first quadrupole to let only ions of one m/z (typically the molecular ion) pass on to the second quadrupole. This so called precursor ions is then fragmented into product ions in the second quadrupole and the third quadrupole is set to allow only fragments of one m/z pass on to the detector. The m/z of the precursor (first

quadrupole) and the selected product (third quadrupole) is often referred to as the MRM-transition.

Figure 7 is a schematic of a triple quadrupole used in MRM mode.

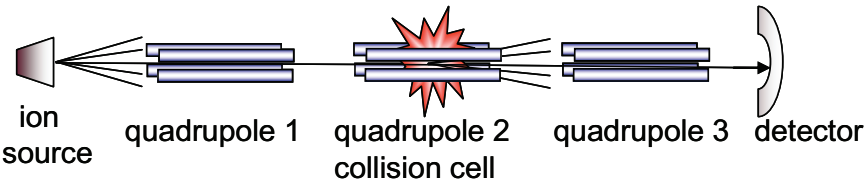


Figure 7. Schematic of a triple quadrupole mass spectrometer in multiple reaction monitoring (MRM)-mode.

Liquid chromatography-tandem mass spectrometry

The combination of LC and MSMS is a powerful hybrid analytical method, and the growth in applications of this technique is exponential. For LC-MSMS analysis of compounds in biological samples, an isotopically labeled internal standard is typically added followed by some sort of sample clean up to remove proteins or reduce ionic strength. The analyte is then either analyzed directly or derivatized and separated from interfering compounds on a reversed phase column. MS-detection is often performed in MRM-mode.

During the work on this doctoral thesis, one method for determination of oxalic acid in urine using LC-MSMS was published. Urine was analyzed after only dilution and addition of $^{13}C_2$ oxalic acid and injected on a reversed phase/weak anion exchange column without prior derivatization. The native oxalic acid and isotopically labeled internal standard was detected by MRM in negative ionization mode. However, the method was not sensitive enough for plasma determinations as the lower limit of quantitation was 100 µmol/L[219].

Objectives of the Thesis

Reliable quantitation of oxalic acid in body fluids is of great importance in several medical conditions, both inherited and acquired. For laboratory diagnosis of the inborn errors of metabolism primary hyperoxaluria type I and II (PH I and PH II), elevated concentration of oxalic acid is the biochemical hallmark. Environmental or secondary hyperoxaluria can be caused by increased ingestion and/or absorption of dietary oxalic acid, intestinal disease or surgery or alterations in intestinal bacterial flora. Oxalic acid is eliminated from the body by the kidneys, and excess oxalic acid can precipitate as calcium oxalate and cause tissue damage. Therefore, end-stage renal failure can be expected to involve a risk of elevated body burden of oxalic acid, regardless of the underlying disorder.

Despite the importance of quantitation of oxalic acid, no analytical method has been available with satisfactory performance characteristic for both plasma and urine analysis to be used in a routine clinical lab. The combination of different pre analytical and analytical factors has lead to an extensive variation in normal ranges of oxalic acid reported using different analytical procedures. For urine analysis of oxalic acid, the use of 24-h urine collection has traditionally been performed. Especially in children this is cumbersome and non-compliance is common.

For diagnosis of PH, a normal or close to normal 24-h urinary excretion of oxalic acid may be observed with deteriorating kidney function. However, the oxalic acid/creatinine ratio remains elevated.

The main aim of the thesis was to (1) develop a state of the art method for determination of oxalic acid for analysis of both plasma and urine and (2) estimate the normal range of the metabolite in a healthy population, to (3) gain knowledge of oxalic acid dynamics following kidney transplantation in non-PH patients and (4) to investigate the diurnal excretion patter of oxalic acid to assess the usefulness of relating oxalic acid to creatinine in spot urine as an alternative to the traditional 24 h-urine collections.

Specific objectives of the projects:

Paper I

The aim of this study was to develop a reliable and user friendly method for oxalic acid determination in plasma and to evaluate the performance characteristics of the method. To be able to recognize elevated levels of oxalic acid, the normal range of the metabolite in a healthy population also had to be established. Further, the impact on pre analytical factors on the quantitative results was investigated including delayed processing and variable storage conditions.

Paper II

It is well recognized that in PH patients calciumoxalate deposits in the kidneys can lead to organ damage and eventually ESRF. Following kidney transplantation in PH, resolubilization of oxalic acid from extra-renal deposits may result in recurrence of kidney failure and repeated kidney transplantation may therefore bee needed. Theoretically, oxalic acid may be harmful in all patients with ESRF regardless of the underlying disorder. Elevated levels of plasma oxalic acid in non-PH patients with ESRF have been reported. However, little has been known about the oxalic acid dynamics following kidney transplantation in these patients. The aim of this study was therefore (1) to find out to what extent plasma oxalic acid was elevated in non-PH kidney transplant recipients and the effect of kidney transplantation on plasma oxalic acid levels and (2) to try and identify determinants of plasma oxalic acid following kidney transplantation by collecting and analyzing data on relevant demographic and laboratory parameters.

Paper III

The aim of this study was to evaluate the usefulness of the plasma method for analysis of oxalic acid in urine. Secondly, as the collection of 24-h urine is cumbersome it was desirable to investigate weather 24-h urine collections could be substituted with the much simpler spot urine collection. The establishment of precursory reference intervals for spot-urine oxalic acid/creatinine-ratio in both children and adult, without diet restrictions was investigated.

Methods

Paper I

LC-MSMS is a powerful analytical tool routinely used in hospital laboratories for quantitation of a number of metabolites in biological samples. The use of MSMS in MRM mode allows detection with a high degree of confidence. The use of isotopically labeled $^{13}C_2$-oxalic acid as internal standard can compensate for eventual sample loss during sample preparation and is easily separated from the native compound in MRM. Therefore, LC-MSMS in MRM mode with $^{13}C_2$ oxalic acid as internal standard was the natural choice as a starting point for method development. Some sort of sample preparation is needed to reduce the complexity of the sample and avoid clogging of the chromatographic column. Being a strong acid, oxalic acid is readily retained on a strong anion-exchange (SAX) solid phase extraction (SPE) material and as this sample preparation technique can easily be automated it was chosen as the preferred sample preparation procedure. Native oxalic acid can be ionized in negative ionization mode in the mass spectrometer, but the ionization efficiency is low and thus low sensitivity is obtained. Fortunately, oxalic acid is easily derivatized by heating with acid and alcohol, and the resulting di-esters are readily ionized in positive ionization mode in the mass spectrometer. In addition they produce fragments with high abundances that can be used for MRM measurements. Di-esters of different chain lengths were therefore prepared and tested. Compared to the native compound, the di-esters are also much more hydrophobic and thus a reversed phase chromatographic system was adapted for the LC separation.

The method was validated in terms of assessment of limit of quantitation, linearity, within-day repeatability, LC-MSMS repeatability and long-term reproducibility. Absolute recovery during the steps of sample preparation was investigated using a radioactive isotope of oxalic acid, $^{14}C_2$-oxalic acid, and recovery relative to the internal standard was tested.

The impact of delayed processing of plasma after intake of the main contributor to in vitro oxalogenesis, vitamin C, was also assessed. Finally, the impact of storage temperature on long term stability of samples was investigated.

Once the analytical method was validated including establishment of pre-analytical procedure, the normal range of oxalic acid in plasma was established by analyzing samples from healthy individuals. Samples from some PH patients were also analyzed.

Paper II

A total of 213 patients admitted for kidney transplantation at Oslo University hospital Rikshospitalet were recruited during a period of 15 months. One patient with a plasma oxalic acid of 157 μmol/L at time of transplantation was later found to have PH1 and was excluded from the study. Relevant demographic data for all patients were collected. The mean recipient and donor age were 51 and 49 years, respectively, and 64 % of the patients were men. Approximately half of the patients received a transplant from a live donor. One third of the patients were transplanted preemptively (did not receive dialysis treatment at time of transplantation) and 14 % had non primary function (did receive dialysis treatment up to 10 weeks follow up). The median time in renal replacement therapy (dialysis treatment) prior to transplantation was one year. Samples for oxalic acid were collected at the same time as samples for standard laboratory evaluation both pre transplantation and at 10 weeks follow up. However, to avoid oxalogenesis, the vials with plasma for oxalic acid determination were processed quickly after collection and stored at -70 if not assayed immediately. Data on plasma creatinine, calcium, phosphate, albumin, hemoglobin, and urea were also collected at both time points and GFR at follow up. Adequate samples for oxalic acid were obtained for 99 and 167 out of the 213 at transplantation and follow up, respectively. Univariate relationships were evaluated by means of Spearman's correlation coefficients. The determinants of oxalic acid were investigated by multivariate regression analysis, and values for plasma oxalic acid, creatinine, urea and PTH had to be log transformed to be normally distributed before entering the statistical model.

Paper III

The analytical method from paper I was adaptable for urine analysis only after dilution of urine 1:9 with water. Within batch precision was tested, and relative and absolute recoveries were investigated using the same protocol as for plasma. As precipitation of calciumoxalate is highly pH dependant, absolute recoveries of oxalic acid at different sample pH were also assessed. An enzymatic kit based on oxalate oxidase is commercially available for urine analysis of oxalic acid. The current method was compared to the kit by analyzing the same urine samples with both methods and comparing the results by t-test on ratios. To investigate the possible use of spot urine oxalic acid/creatinine-ratio, the diurnal patterns of

oxalic acid and creatinine were investigated in a group of healthy individuals who collected every void during 24 h in separate containers.

Finally, spot urines from children presumably healthy with respect to hyperoxaluria disorders and healthy adult males and females were collected and analyzed and precursory reference intervals for oxalic acid /creatinine-ratio estimated by linear regression.

Main findings

Paper I

Derivatization of oxalic acid into di-butyl esters of three different chain lengths were initially tested (propyl, butyl, and pentyl). The di-butyl ester gave the most satisfactory result and was used for further method development. In the MS, the di-butyl ester of oxalic acid and the internal standard $^{13}C_2$-oxalic acid produced molecular ions corresponding to the protonated molecules (MH+) at m/z 203 and 205, respectively. The main fragments produced in the collision cell were due to the loss of one butyl group (m/z 57). The transition from the molecular ion to the butyl fragment was chosen as MRM transitions; 205.1 – 57.1 and 203.1-57.1 for the internal standard and native compound, respectively. Satisfactory sample cleanup was obtained by SAX-SPE and elution with acidified organic solvent. Initially, hydrochloric acid in methanol was used, but due to the potential formation of methyl esters in the proceeding derivatization (if the solvent was not removed properly), acetonitrile was instead used. Chromatographic performance was obtained using a regular C-8 reverse phase column and a mobile phase of 60 % acetonitrile in water. The assay was found to be linear up to 200 μmol/L using aqueous standards, and the limit of quantitation in plasma was 0.5 μmol/L. For within-day repeatability, an average coefficient of variation (CV) of 4.5 and 6.9% was found for an aqueous standard and for plasma, respectively. Due to oxalogenesis, a real plasma sample could not be implemented as long-term quality control, and the use of artificial plasma was not compatible with the chromatographic system. As an alternative, an aqueous standard was implemented as quality control, and over a 3 moths period a CV of 5% was found.

Relative recovery, as tested by spiking plasma with 5-200 μmol/L oxalic acid, resulted in a satisfactory recovery of over 90% at all concentrations tested. Absolute recovery, as tested by addition of radioactive oxalic acid, revealed that sample loss was minimal during SPE and the first evaporation step. In contrast, the sample loss during the second evaporation step, after derivatization, was found to be substantial and only about 25% of the oxalic acid originally present in the sample was recovered. These experiments clearly showed that the dibutyl-ester was far more volatile than the native compound and that the second evaporation step had to be performed under very mild conditions.

The level of oxalogenesis in fresh plasma was tested with and without vitamin C. Significantly higher oxalogenesis was observed both when vitamin C was ingested and

when it was added directly to the sample. The results revealed that samples must be processed without delay following collection to avoid significant oxalogenesis.
The effect of storage time up to eight weeks on samples stored at -20 and -70°C was also investigated. Oxalogenesis was observed after storage at both temperatures, and the absolute increase measured was found to be negatively related to the initial value.
Finally, the normal range of oxalic acid in plasma was found by analyzing plasma from 67 apparently healthy adult men and women without any dietary restrictions. No statistically significantly differences in plasma oxalic acid between the genders or age dependency were found. The data were log-normally distributed and by regression analysis the normal range was found to be 3-11 μmol/L, which is in the middle range of normal ranges reported in the literature. In three PH-patients, plasma concentrations in the range 50-170 μmol/L were found.

Paper II

At transplantation, 98 % of the plasma oxalic acid concentrations were above the upper reference limit. The saturation limit for calcium oxalate in plasma has been reported to be in the range 35-40 μmol/L meaning that at higher levels there is a risk for precipitation. Almost 40% of the patients had plasma levels above 40 μmol/L. At 10 weeks post transplant, still more than one third of the patients had oxalic acid levels above the upper reference limit. In patients with elevated oxalic acid at follow up, a higher average creatinine value (144 μmol/L) compared to those with oxalic acid within the normal range (108 μmol/L) was found. The correlation analysis of the data at transplantation revealed that oxalic acid was statistically significantly correlated both to preemptive transplantation, phosphate and creatinine. However, in the subsequent multiple regression analysis only preemptive transplantation and creatinine retained significance. This finding supports the hypothesis that oxalic acid concentration increases with declining kidney function. At 10 week follow up, both phosphate, creatinine, urea, albumin, and hemoglobin at 10 weeks, recipient and donor age, rejection episodes, GFR, and baseline PTH were found to correlate significantly with oxalic acid. Although all these parameters were entered into the subsequent regression model, only donor age, creatinine and GFR were retained as significant determinators of oxalic acid. These results revealed that the plasma level of oxalic acid at transplantation was not important for the reduction in plasma oxalic acid

observed following transplantation, but that establishment of adequate kidney function is the major determinant for excretion of the excess oxalic acid.

Paper III

The method originally developed for plasma was found to work excellent for urine after only dilution of the sample. The within batch CV did not exceed 6.9 % at any concentration tested (4.5 – 105 μmol/L in diluted sample). A satisfactory mean relative recovery of 97% was found for urine spiked with 5-200 μmol/L oxalic acid. In regard to absolute recovery, the same pattern as for plasma was observed; minor loss of oxalic acid during SPE and the first evaporation step but significant loss of the derivatized oxalic acid in the second evaporation step emphasizing the importance of evaporation using low gas flow and moderate temperature. Extreme acidification of urine resulted in higher sample loss during SPE, but the absolute recovery for the whole analytical procedure did not show any strong pH dependence. From the analysis of 21 urines evenly distributed from 21 -1025 μmol/L by both the current method and the enzymatic kit, it was found that the current method gave 7.9 % higher values than the enzymatic kit, independent of the oxalic acid concentration. Quantitation of oxalic acid and creatinine in every void during 24 hours from 9 healthy adult volunteers (giving a total of 57 samples) revealed no significant time dependent variation in oxalic acid/creatinine-ratio. These data suggested that random spot-urines could be used as an alternative to timed urine collection. The variation of oxalic acid, creatinine and oxalic acid/creatinine-ratio in 47 children (age 1 month-17years) presumably healthy with respect to hyperoxaluria disorders revealed that oxalic acid excretion did not differ significantly between boys and girls. However, the strong age dependency of creatinine excretion resulted in a decrease in oxalic acid/creatinine-ratio with age. In 56 healthy adults (age 24-76 years) a slightly decreasing oxalic acid excretion with age was observed, but regression analysis revealed no age dependence in oxalic acid/creatinine-ratio. Men were found to have slightly higher oxalic acid excretion compared to women, but as a result of the significantly higher creatinine excretion in men, this gender had significantly lower oxalic acid/creatinine -ratio. Using linear regression, precursory reference intervals for children and adults were calculated as 95% central intervals. For children under one year age, 78-160 μmol oxalic acid/mmol creatinine was found as the normal range, dropping to 17-34 μmol oxalic acid/mmol creatinine at age 17 years which concurred with the values found in the adult population.

Conclusions of the Thesis

A state of the art SPE-LC-MSMS method for determination of oxalic acid in plasma and urine has been developed, characterized and validated. The method is based on an initial addition of an isotopically labeled internal standard $^{13}C_2$-oxalic acid followed by a sample cleanup step using a strong anion exchange resin. Oxalic acid is retained on the material while neutral and positively charged interferences are not thus reducing the complexity of the resulting sample obtained when the analyte is eluted off the resin with acidic acetonitrile. After evaporated to dryness, derivatization is performed with addition of acidic butanol and heating for 15 minutes. The resulting di-butyl esters are volatile and removal of excess derivatization reagent must be performed under mild conditions to avoid unacceptable sample loss. The analyte is separated from the main matrix components by reversed phase LC connected to a triple quadrupole MSMS, and oxalic acid and the internal standard quantified using MRM. Using the developed method, the normal range of oxalic acid in plasma following an unrestricted diet was found to be 3-11 μmol/L, which is in the middle range of normal ranges reported in the literature. Vitamin C results in significant oxalogenesis in plasma if samples are left at room temperature for extended periods of time. After collection, samples must therefore be assayed latest within one hour or stored at -70°C. Even in frozen samples oxalogenesis occurs, and samples should therefore be stored for no longer than one week before analysis to obtain reliable results.

In patients with end-stage renal failure not due to PH and who were admitted for kidney transplantation, significantly elevated plasma levels of oxalic acid was found in 98% out of the 212 patients included in the study. More than one third of the patients had oxalic acid levels above the saturation limit for calciumoxalate in plasma, indicating a potential risk of oxalosis.

The independent determinants of oxalic acid at transplantation were found to be plasma creatinine and preemptive transplantation, pointing to the important role of the kidney in removing oxalic acid from the body; when the kidneys ability to excrete oxalic acid drops, the plasma level increases.

As expected following kidney transplantation the oxalic acid levels dropped substantially and to concentrations below the saturation limit. However, almost 40% of the patients still had oxalic acid levels above the upper normal limit 10 weeks after transplantation. The level of oxalic acid before transplantation turned out not to be of importance for the subsequent

reduction. The independent determinants of oxalic acid after kidney transplantation were found to be GFR, creatinine and donor age. Both plasma creatinine and GFR are measures of kidney function, and both creatinine and oxalate and GFR at 10 weeks follow up were significantly correlated. A possible explanation for the fact that both creatinine and GFR were retained in the subsequent regression model can be that the two parameters have different accuracy with respect to predict oxalate levels at different levels of the two parameters.

Three of the patients had extremely elevated oxalic acid levels before transplantation. In one, the plasma level remained elevated after transplantation and the patient was later diagnosed with PH. In the other two, plasma oxalic acid dropped to close to normal levels after transplantation. One had a history of gastro intestinal surgery and kidney stones making enteric hyperoxaluria a possible explanation of the elevated oxalic acid.

For quantitation of oxalic acid in urine, no significant time dependency in the ratio of oxalic acid excretion relative to creatinine excretion was found. This indicates that creatinine and oxalic acid are treated similar by the kidney and that spot urine can be used as an alternative to 24-h urine. However, ingestion of a meal extreme in oxalic acid or its precursors may theoretically lead to transient elevated oxalic acid/creatinine-ratio that do not reflect the patients true oxalate status. In healthy children, the oxalic acid/creatinine-ratio was the same for boys and girls and decreased from birth and up to age 17 years due to the increase in creatinine excretion with age. In adults, no age dependency was found but men excreted more of both oxalic acid and creatinine leading to a slightly lower oxalic acid/creatinine-ratio in this gender.

Future Perspectives

In addition to the elevated oxalic acid found in PH, elevated glycolic acid is found in PH1 and L-glyceric acid in PH2. Glyceric acid is normally not detectable in plasma or urine. In contrast to glycolic and glyceric acid, oxalic acid is elevated in several other medical conditions than PH. Thus, quantitation of glycolic and glyceric acid together with oxalic acid may provide important information in laboratory diagnosis of PH. Glycolic acid (HO-CO-CH2-OH mw 76.05 g/mol) and glyceric acid (HO-CH_2-CH (OH)-CO-OH, mw 106.08 g/mol) both contains one acid group and should in theory be suitable for anion exchange SPE, esterification and reversed phase LC like oxalic acid. Isotopically labeled analogs are available that can be used for reliable quantitation by MSMS in MRM-mode.

Thus, the developed method for oxalic acid might be suited for determination of glyceric and glycolic acid in both plasma and urine as well, and this possibility should be investigated. If successful, the next step would be to estimate the normal range of glycolic acid in a healthy population.

For spot urine analysis, more samples from healthy individuals should be analyzed to confirm the precursory reference intervals. With more data it will also be easier to conclude whether the slightly different oxalic acid/creatinine-ratios in men compared to women observed should result in the use of gender specific reference limits.

As the current work is based on measurement of oxalic acid without any special dietary restrictions, it would also be interesting to test the effect in plasma and urine of ingestion of meals with can be expected to cause temporary increase in oxalic acid.

The study on plasma oxalic acid following kidney transplantation in non PH patients revealed that the majority of these patients have pre-transplantation oxalic acid levels that at least in theory induce a risk of precipitation of calciumoxalate. Available reports on the magnitude and role of oxalosis in renal failure are not conclusive, and relatively little is known about oxalosis following kidney transplantation. The saturation of plasma with calcium oxalate may be only one out of several factors that leads to oxalosis. By heterotropic cardiac transplantation in rats it has been showed that the occurrence of prior tissue damage could represent a crucial predisposing factor in the deposition of calcium oxalate crystals[137]. Following these observations, one can only speculate on the possible impact of kidney tissue damage on renal oxalosis.

Whether the elevated plasma oxalic acid seen in the kidney transplant patients has long term consequences on graft function is not known today. Perhaps there is a greater risk for precipitation of calcium oxalate in a transplanted kidney.

An expanded study involving assessment of oxalic acid and other relevant laboratory and clinical data, in combination with biopsy studies to reveal the eventual presence of calcium oxalate deposits would have the potential to expand our knowledge of the role of oxalic acid in renal failure.

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Article 1 is removed.

Article 2

PLASMA OXALATE FOLLOWING KIDNEY TRANSPLANTATION IN PATIENTS WITHOUT PRIMARY HYPEROXALURIA

Katja B P Elgstoen [1,3], Linda Flaa Johnsen [2], Berit Woldseth [1], Lars Morkrid [1,3], Anders Hartmann [2,3].

[1]Department of Medical Biochemistry, Rikshospitalet University Hospital, Oslo, Norway

[2]Department of Medicine, Rikshospitalet University Hospital, Oslo, Norway

[3]University of Oslo, Norway

Abstract

Background: Patients with primary hyperoxaluria may need repeated kidney transplants due to damage from oxalic acid (oxalate) deposits. However, oxalate may also be potentially harmful in all transplant recipients. Determinants of oxalate following transplantation have not been well studied.

Methods: 212 recipients admitted for transplantation were included in the study. Blood samples for measurement of oxalate and other relevant laboratory parameters were collected at baseline and subsequently 10 weeks after transplantation. We examined the bivariate association between plasma oxalate at transplantation and preemptive transplantation, time on dialysis, recipient age, creatinine, urea, phosphate, hemoglobin, PTH, albumin and calcium. Oxalate 10 weeks after transplantation was tested likewise including also laboratory parameters at baseline, primary non-function, rejection episodes, live versus deceased donor, donor age, and GFR at follow-up.

Results: Median plasma oxalate concentration at transplantation was 35.0 µmol/L (95% confidence interval(CI) = 10.4- 93.9) and 98% of the values were above normal limits (2.6-11.0). Oxalate concentration after 10 weeks was 9.0 µmol/L (4.0- 25.5), still 37% being above upper normal value.

Multiple regression analysis revealed established dialysis treatment (p= 0,002) and creatinine (p<0,000001) as independent positive determinants of oxalate at transplantation. Oxalate at 10 weeks was negatively associated to ^{51}Cr-EDTA absolute GFR (p= 0,023) and positively to donor age (p=0,027) and plasma creatinine at 10 weeks (p= 0,03).

Conclusion: At transplantation plasma oxalate was on average 3 times increased and above the upper normal limit in 98% of patients and were still above normal in 37% after 10 weeks.

The reduction after 10 weeks is determined by GFR and donor age. Whether increased plasma oxalate following kidney transplantation may have long-term consequences needs further study.

Key words : dialysis, end stage renal failure, kidney transplantation, oxalate.

Short summary:

Plasma oxalate concentration and determinants of oxalate at time of kidney transplantation and at 10 weeks follow up were examined. Oxalate values were increased and above normal limits in 98% and on average 7-fold increased compared to normal. After 10 weeks still 37% had oxalate above the upper normal value. Multiple regression analysis revealed established dialysis treatment and creatinine as independent positive determinants of oxalate at transplantation, while oxalate at 10 weeks was found to be negatively associated to ^{51}Cr-EDTA GFR and positively to donor age and plasma creatinine at 10 weeks.

Introduction

Primary hyperoxaluria (PH) includes two rare, well characterized autosomal reccesive diseases: primary hyperoxaluria type 1 (PH1) and primary hyperoxaluria type 2 (PH2). PH1 is caused by deficiency of the liver specific peroxisomal enzyme alanine:glyoxylate aminotransferase and PH2 by a deficiency of the cytosolic enzyme glyoxylate reductase/ D-glycerate dehydrogenase[1]. Both enzyme deficiencies results in an excess production of oxalic acid (oxalate), a metabolic end product that is excreted in urine. Oxalate binds to calcium forming calciumoxalate that is virtually insoluble at physiological pH. In PH1, progressive deposition of calciumoxalate often leads to deteriorating kidney function and can result in end stage renal disease [2]. The biochemical hallmark of PH is severe hyperoxaluria, but with deteriorating kidney function hyperoxalemia develops. Kidney transplantation is not regarded as a successful treatment option in PH1 as in most cases the disease rapidly leads to oxalate deposits in the transplant and subsequent graft loss is common [2;3]. Therefore a combined liver and kidney transplant has emerged as therapy of choice since the metabolic defect is then also restored [3]. However, still recurrence of deposits in the kidney is a problem due to high accumulated oxalate stores which can result in high plasma levels of oxalate [4]. PH2 causes similar, but usually milder symptoms. Non-hereditary elevated plasma oxalate due to increased enteric absorption may occur with diseases of the intestine and after bariatric surgery [5]. This may also lead to kidney oxalate deposition and eventually renal failure and may also cause graft loss after kidney transplantation [6]. Another major cause of oxalate retention is kidney failure per se, since the main excretion pathway of oxalate is glomerular filtration and secretion [7]. When end-stage renal patients are successfully treated with a kidney transplant, stored oxalate may be excreted by the graft and potentially harm the transplant as indicated in several biopsy studies [8-10]. The retention of oxalate in non-hyperoxaluria patients

with end-stage renal failure has not been well studied [11]. We therefore initiated a prospective study and measured plasma levels of oxalate at the time of kidney transplantation and early post-transplant to assess the magnitude of hyperoxalemia in a larger series of patients. We also aimed to address potential determinants of plasma oxalate.

Subjects and Methods

Study design

In this single centre prospective study we measured plasma oxalate at arrival in the transplant center and also in a stable phase on average 10 weeks after transplantation in altogether 213 kidney recipients. The patients were recruited from February 2004 throughout May 2005. Due to lack of back-up for pre-analytical laboratory handling at all times, baseline samples could not be obtained in all cases. Samples for oxalate measurement that were not collected or stored correctly were excluded from the study (see "Plasma oxalate measurements"). Out of 213 patients, we received adequate oxalate samples at baseline for 100 patients and for 168 patients at 10 weeks. One patient with a plasma oxalate value of 157 μmol/L at transplantation was later found to have primary hyperoxaluria type 1 and was excluded from the study leaving 212 for observation in the present study. A test for homogeneity (ANOVA) was performed after subdivison into three separate groups (I = oxalate data obtained only at transplantation, n = 45; II = oxalate data obtained both at baseline and follow-up, n = 54; III = oxalate data only available at follow-up, n = 113). The test did not reveal any significant differences between these groups for all relevant demographic and laboratory parameters. To obtain meaningful analysis of determinants of oxalate at baseline and at follow-up we examined only 2 groups, those with oxalate data at transplantation (n= 99) and those with oxalate data at follow-up (n=167). Between these two groups, the only difference found was

a higher proportion of live donor and preemptive transplants in the baseline cohort than those tested at 10 weeks, probably due to better availability of blood samples in these elective patients.

The patients generally received a triple based immunosuppressive regimen comprising CNI inhibitor (CsA 80%, tacrolimus 20%), MMF and prednisolone.

The demographic data of the overall cohort is shown in Table 1. All patients signed an informed consent form and the study was approved by the Regional Ethics Committee.

Statistics

Univariate relationships were examined by means of Spearman's correlation coefficients. To ensure that continuous laboratory data were normally distributed before entering multivariate regression analyses (backward and forward procedures), values for plasma creatinine, urea, oxalic acid and PTH were log transformed. SPSS version 16 was used for statistical calculations. Two-tailed tests were applied, and significance level=0.05 was adapted.

Plasma oxalate measurements

The plasma oxalate measurements were done on fresh samples of heparinized plasma. To avoid *in vitro* oxalogenesis (non-enzymatic conversion of plasma constituents into oxalate), resulting in falsely high plasma oxalate, efforts were made to ensure optimum pre-analytical conditions; after collection, the samples were centrifuged without delay and the plasma was assayed within one hour or stored at -70°C for no longer than one week. All samples were assayed in duplicate. The oxalate was measured by means of solid phase extraction followed by derivatization and liquid chromatography – tandem mass spectrometry (LC-MSMS) as recently validated and described in detail [12].

GFR at 10 weeks was measured by plasma disappearance of ^{51}Cr-EDTA and normalized to 1.73m^2 body surface. Hemoglobin was measured on a CELL-DYN 4000 automatic

haematological analyzer (Abbott Diagnostics, CA, USA). All other laboratory parameters were measured by standard procedures on Modular Automatic Analyzer (Roche Diagnostics, Basel, Switzerland). Due to the vast variability in kidney function early after transplantation urinary parameters were not assessed.

Results

Median plasma oxalate concentration at transplantation was 35.0 μmol/L (CI = 10.4- 93.9) with 98% of the values above the upper normal value (reference: 3.0 -11.0)[12]. More than one third of the values (39%) were higher than 40 μmol/L which is considered to represent the saturation limit for calcium oxalate (CaOX)[13]. After 10 weeks oxalate concentration was significantly lower, with a median of 9.0 μmol/L (CI= 4.0- 25.5). Still 37% of the values were above the upper normal limit. In the patients with elevated plasma oxalate at follow up, a mean creatinine of 144 μmol/L (CI: 81-309) was found versus those with plasma oxalate values within normal limits who had a mean creatinine of 108 μmol/L (CI: 57-187).
Two patients had extremely high oxalate values at transplantation that fell from 156 to 10 μmol/L after 10 weeks in one and from 124 to 16 μmol/L in the other.
All oxalate values are depicted in Figure 1. The laboratory and biochemical data at transplantation and after 10 weeks are shown in Table 2.

We examined univariate relationships between plasma oxalate at transplantation and corresponding values for creatinine, urea, phosphate, PTH, albumin, total calcium, hemoglobin, preemptive transplantation, time on dialysis, and recipient age. Oxalate at transplantation was found to be significantly correlated to both preemptive transplantation phosphate, and creatinine but only preemptive transplantation and P- creatinine retained

significance in a subsequent multivariate regression analysis: lg [oxalate at TX] = -1.0471 + 0.8918·log [creatinine] + 0.1136· [preemptive transplantation; no = 1; yes = 0]; P-values for the regression coefficients were 0.0016, < 0.0001, and 0.0021 respectively. Standardized results from the multivariate analysis are shown in Table 3. The relationship between plasma oxalate and plasma creatinine at the time of transplantation is shown in Figure 2. Patients who received preemptive transplantation and patients transplanted after start of dialysis are shown separately. The increase in plasma oxalate with increasing creatinine values is similar in the two patient groups although patients receiving dialysis had oxalate values about 6.5 μmol/L higher than the preemptive patients.

Oxalate at 10 weeks was tested likewise, with all laboratory parameters at 10 weeks, but also including all laboratory parameters at baseline, primary non-function, rejection episodes, live versus deceased donor, donor age and GFR at 10 weeks. Both phosphate, creatinine, albumin, urea, and hemoglobin at 10 weeks, baseline PTH, recipient and donor age, rejection episodes and GFR were found to correlate significantly with oxalate at 10 weeks. In the subsequent multivariate regression analysis, only GFR, creatinine and donor age retained the significance as determinants of oxalate: lg [oxalate at 10 weeks] = 0.9277 – 0.4241.·log [GFR] + 0.002224· donor age + 0.3273·log [creatinine]; P-values = 0.12, 0.02, 0.028 and 0.032 respectively. See also Table 3.

Discussion

This is the first study of a larger cohort of patients addressing plasma oxalate in kidney failure patients before and after transplantation. This is also the first study to address the determinants of plasma oxalate in these patients. The data confirm earlier more limited observations of very high levels of oxalate in patients with end-stage kidney failure [11]. The increase was sevenfold compared with healthy persons. The independent determinants of oxalate in multivariate regression analysis were creatinine and established dialysis treatment, supporting the hypothesis of increasing values with declining kidney function. It has previously been shown that oxalosis, the deposition of CaOX in tissues, can be a complication of chronic renal failure. CaOX crystals have been found both in kidneys and myocardium of these patients at autopsy [14]. Supersaturation of CaOX in plasma occurs when plasma oxalate level raises beyond 40 μmol/L [13]. More than one third of our patients had values beyond this limit at admission for kidney transplantation despite the fact that many had preemptive transplantation implying some residual capacity for renal excretion of oxalate. It was interesting to note that at time of transplantation, two of the patients had oxalate concentrations in the same range as found in the patient who was later diagnosed with primary hyperoxaluria. In one of these patients the elevated plasma oxalate might have been due to enteric hyperoxaluria. Distal parts of ileum and coecum had been removed due to accidental thrombosis 25 years before transplantation and she developed several kidney stones and was transplanted with a live donor a year after start of dialysis. The other patient with grossly elevated plasma oxalate at transplantation had no history of gastrointestinal disease or GI surgery and had a clinical diagnosis of nephrosclerosis. However, at 10 weeks follow up, the two non-PH patients had oxalate values at, or slightly above the upper reference limit, while the PH patient still had oxalate values tree times the upper reference limit (results not shown).

The severity of oxalosis has also been found to be related to the duration of renal insufficiency [14]. However, we could not demonstrate that plasma oxalate concentration at the time of transplantation was significantly associated with the time on renal replacement therapy. Our results might have been biased in patients who had started regular dialysis treatment since it is well recognized that plasma oxalate is readily removed by dialysis [15], and is thus reduced after each treatment session and increases by refilling from extra-vascular stores until the next dialysis session .Therefore the time elapsed since last dialysis session may have influenced the results. We have limited information on the time elapsed from the last dialysis session to the time of blood sampling at admission for transplantation. However, analysis of data from preemptive patients revealed the same relationship between oxalate and creatinine substantiating that increasing retention of oxalate occurs with declining kidney function.

Median plasma oxalate at 10 weeks was 9 μmol/L indicating some 75% reduction of plasma oxalate compared with the values at admission for transplantation. This finding was not surprising but has not previously been reported in the literature. Interestingly more than one third of the patients still had values above the upper reference limit for healthy controls, and one had a value beyond the supersaturation level of 40 μmol/L. This is in contrast to findings in a previous small study of 8 patients undergoing living related kidney transplantation [11] where plasma oxalate was found to be in the normal range three days post transplant although a tendency for higher oxalate levels was observed when compared with controls. The lack of statistical significance might be attributed to the inter-individual variability and the fact that the above mentioned study only comprised eight patients. Episodes of kidney stones were not recorded in any patient. Episodes of urinary tract obstruction were caused by lymphocele or uretheral necrosis, in no case was obstuction caused by stone in the transplant urinary tract.

We demonstrated that kidney function at 10 weeks as measured by plasma ^{51}EDTA GFR (and also plasma creatinine) and donor age were the only determinants of oxalate in multivariate regression analysis. Baseline level of oxalate was not a predictor for the reduction at 10 weeks indicating that the plasma load of oxalate at the time of transplantation is not important for the subsequent reduction in plasma oxalate. By contrast establishing of an adequate kidney function for excretion of the excess oxalate load is obviously of importance. However we could not find that primary non-function of the kidney transplant was of importance as long as an acceptable function was established within the first few weeks.

The measurement of oxalate in biological fluids has been challenging, especially due to *in vitro oxalogenesis*, leading to erroneously high plasma oxalate from the autooxidation of ascorbate into oxalate after sampling and even during assay [16]. Thus, to obtain reliable quantitative results, samples for oxalate determination must be processed expeditiously.

We found that 37% of the patients studied had plasma-oxalate above the upper reference limit after 10 weeks. This could at least theoretically have been attributed to oxalogenesis, if sample collection and pretreatment had not been standardized. However, all samples included in this study have been handled according to protocol. The few samples that had not been correctly treated (e.g left at room temperature after collection etc) were excluded from the present analysis.

Strengths of the study

The study is prospectively performed and the analyses are well validated [12]. This is the first study of a larger cohort of patients with kidney failure measuring plasma oxalate before and

after transplantation. This is also the first study of the determinants of plasma oxalate in these patients.

Limitations

The plasma concentrations of oxalate may not be representative of the tissue damage observed in biopsy and autopsy studies. The importance of the hyperoxalemia in these patients remains obscure and needs follow-up studies of clinical endpoints or simultaneous protocol biopsies addressing oxalate deposits in the kidney transplants.

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Conflict of interest statement:

We have had no involvements that might raise the question of bias in the work reported or in the conclusions, implications, or opinions stated.

Table 1. Demographic and transplant data for 212 kidney transplant patients

Variables	
Number of patients	212
Recipient age	51 (11-81)
Sex male/female (%)	135/77 (64/36)
Donor age, years (range)	49 (1-77)
Live donor transplants (%)	118 (49)
Preemptive transplantation (%)	53 (25)
Dialysis time, median in months (range)	12,8 (0 – 369)
Non primary function (%)	29 (14)

Table 2. Laboratory data for 212 kidney transplant patients at transplantation and after 10 weeks.

	At TX				After 10 weeks				Ref. interval
	N	Median	95% central interval	% below lower/ above upper ref. limit	***N***	Median	95% central interval	% below lower/ above upper ref. limit	
P-oxalate (µmol/L)	99	35	10.4 – 93.9	0/98	167	9	4.0 – 25.5	0/37	3 – 11
P-creatinine (µmol/L)	212				167				
♀>15y:		597	284 – 968	0 / 100		95	54 – 163	0 / 60	50 – 90
♂>15y:		619	252 – 1110	0 / 100		122	64 – 303	0 / 72	60 – 105
P-Calcium (mmol/L)	212	2.38	2.01 – 2.78	10 / 18	167	2.41	2.05 – 2.83	5 / 23	2.15 – 2.51
P-Phosphate (mmol/L)	212				167				
♀>16y:		1.7	0.8 – 2.81	0 / 63		0.8	0.4 – 1.29	60 / 0	0.9 – 1.5
♂ 16-49 y:		1.75	0.66 – 3.1	0 / 50		0.9	0.6 – 1.28	25 / 0	0.8 – 1.7
♂≥50y:		1.6	0.89 – 2.55	0 / 60		0.8	0.39 – 1.40	28 / 0	0.8 – 1.4
P-Albumin (g/L)	211				167				
18-39y		42	33.8 – 47	6 / 0		42	34.4 – 46.9	2 / 0	36 – 48
40-69y		40.5	31 – 47	12 / 8		40	32.0 – 44.0	7 / 0	36 – 45
≥70y:		40	32 – 46	11 / 6		38	26.0 – 40	7 / 0	34 – 45
P-Urea (mmol/L)	212				167				
♀ 18-49y:		22.5	9.16 – 45.6	0 / 100		8.55	3.5 – 15.9	0 / 61	2.6 – 6.4
♀ ≥50y:		17.9	7.5 – 34.1	0 / 100		9.35	3.20 – 23.10	0 / 65	3.1 – 7.9
♂ 18-49 y:		19.2	8.72 – 36.8	0 / 100		8.45	2.75 – 16.17	0 / 54	3.2 – 8.1
♂≥50y:		19.3	5.88 – 36.58	0 / 96		11.9	3.83 – 31.24	0 / 70	3.5 – 8.10

Table 3. Multivariate regression analysis of oxalate at transplantation and after 10 weeks.

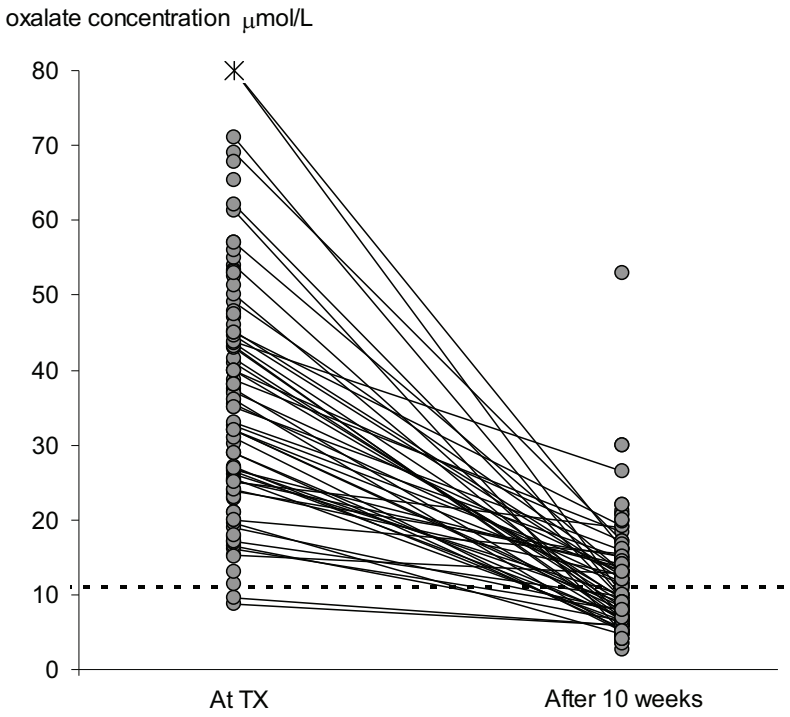
	At TX		After 10 weeks	
Variables	Standardized coefficients	*p*-value	Standardized coefficients	*p* -value
Preemptive transplantation	0.243	0.0021		NS
Recipient age		NS		NS
Donor age		NA	0.167	0.028
Rejection		NA		NS
GFR		NA	-0.248	0.02
P-creatinine [a,b]	0.591 [a]	<0.0001	0.229 [b]	0.03
P-phosphate [a,b]		NS		NS
P-albumin [b]		NS		NS
P-urea [b]		NS		NS
P-oxalate [a]		NS		NS

NA= not applicable; NS,not statistically significant; [a] At transplantation; [b] After 10 weeks

Legend to Figure 1.

All plasma oxalate measurements at transplantation (n=99) and after 10 weeks (n=167). The horisontal dashed line indicates the upper normal reference limit for plasma oxalate. Two exceptionally high values at baseline (124 and 156 μmol/L) are indicated by an asterix (∗). Paired observations in the same patient are connected with lines.

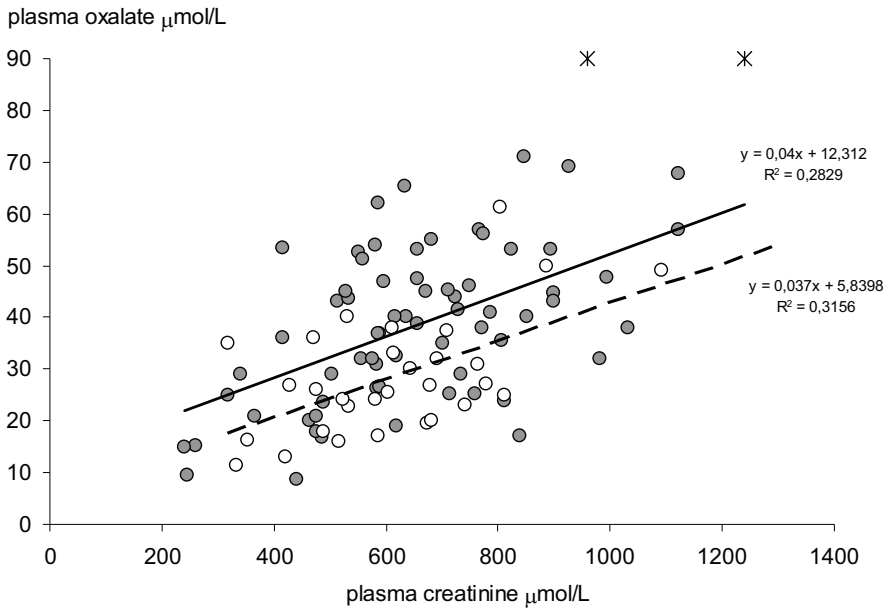
Figure 1.



Legend to Figure 2:

Variation of plasma oxalate with plasma creatinine at the time of transplantation (● = patients on dialysis, ○ = preemptive patients). Trend lines (solid for dialysis and dotted for preemptive), with formulas and corresponding correlation coefficients are shown.
Two exceptionally high oxalate values (124 and 156 μmol/L) are indicated by an asterix (∗).

Figure 2:



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Article 3 is removed.