Primary hyperoxaluria type 1: clinical, genetic and biochemical studies
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Quantification of endogenous oxalate production in patients with primary hyperoxaluria type 1: a stable isotope method

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Background Plasma oxalate concentration and urinary oxalate excretion have been shown inaccurate parameters to monitor endogenous oxalate production (EOP) in patients with primary hyperoxaluria type 1 (PH1) and renal insufficiency. A reliable parameter is needed in these patients to evaluate the efficacy of therapeutic interventions. Therefore, we devised a stable isotope oxalate tracer method to quantify the rate of appearance of oxalate (Ra oxalate).

Methods Three adult patients with PH1 and three adult control subjects, all with a preserved renal function, were given a primed continuous infusion of [1,2-13C]sodium-oxalate lasting eight hrs. Every hour a blood sample was drawn and acidified to determine plasma [1,2-13C]oxalate enrichment. During tracer infusion all urine of the subjects was collected to determine urinary oxalate excretion. Isotopic equilibrium was reached after 360 min of tracer infusion in the patients and after 120 min in the control subjects.

Results Ra oxalate calculated from plasma [1,2-13C]oxalate enrichment at isotopic equilibrium was 0.031, 0.029 and 0.024 μmol/kg-min in patients 1, 2 and 3, and 0.015, 0.017 and 0.016 μmol/kg-min in control subjects 1, 2 and 3, respectively. Urinary oxalate excretion was well reflected by Ra oxalate in all three patients, but was lower than Ra oxalate in controls. This suggests overestimation of Ra oxalate in control subjects, possibly due to in vitro oxalogenesis despite adequate plasma acidification.

Conclusion Ra oxalate is accurately determined in patients with PH1 using our stable isotope method and may be used to monitor therapeutic interventions in patients with PH1 and renal insufficiency.
INTRODUCTION

In primary hyperoxaluria type 1 (PH1) the liver specific peroxisomal enzyme alanine:glyoxylate aminotransferase (AGT; EC 2.6.1.44), which catalyzes the transamination of glyoxylate to glycine, is either deficient or mistargeted to mitochondria instead of peroxisomes (1). Alternatively, glyoxylate can be oxidized to oxalate, a non-functional end metabolite of the glycine oxidative pathway, which is then excreted in urine. Therefore, the deficiency or mistargeting of AGT causes an excess production of oxalate in patients with PH1, which may result in the formation of a highly insoluble calcium-oxalate salt (2). This causes crystalluria, renal stone formation and nephrocalcinosis with a high risk for renal insufficiency and eventually end-stage renal disease (3,4). As a consequence of renal insufficiency calcium-oxalate accumulates in other tissues and organs, resulting in systemic oxalosis, which may finally severely damage organs such as heart, eyes, bone and skin (5). The process of systemic oxalosis starts when glomerular filtration rate (GFR) falls below 60 ml/min·1.73 m² (6).

Only patients with specific gene mutations leading to Gly170Arg or Phe152Ile substitution in the AGXT gene encoding AGT have been shown to respond to pyridoxine supplementation with a decrease of urinary oxalate excretion, probably because pyridoxal-5-phosphate is an essential co-factor for AGT (7,8). Currently, the only therapeutic option for the other patients with PH1 is a liver transplantation in order to eliminate the enzymatic defect of AGT, often combined with a kidney transplantation to restore renal function. However, this procedure has significant morbidity and mortality (9). Therefore, new therapeutic strategies are needed aimed at reducing endogenous oxalate production (EOP) in patients with PH1. Directions of interest include pharmacological interventions in the glyoxylate pathway, hepatocyte cell transplantation, recombinant gene therapy and colonic degradation of endogenous oxalate by Oxalobacter formigenes (10,11).

In order to evaluate therapy efficacy an appropriate marker is needed. It has been shown that plasma oxalate concentration may remain elevated up to years after liver transplantation, due to the wash-out of accumulated oxalate in tissues (9,12). Therefore, plasma oxalate concentration is not a reliable marker for EOP in PH1, as is urinary oxalate excretion in patients with renal insufficiency. Even in patients with PH1 and a preserved renal function, elevated levels of plasma oxalate may mask the efficacy of treatment due to sustained hyperoxaluria. This is emphasized by a recent study in which no effect of OTZ, a potential glyoxylate lowering agent, on urinary oxalate excretion could be detected in two patients with PH1 (13), whereas this effect was demonstrated in healthy individuals (14).
The determination of the rate of appearance of oxalate (Rₐ oxalate) in plasma, reflecting both EOP and oxalate wash-out from tissues, should be a more accurate parameter to evaluate therapy efficacy in patients with PH1 and renal insufficiency. Several studies have quantified oxalate production in patients with PH1 using ¹⁴C labeled oxalate (15-18), but this method is ethically not applicable in clinical practice because of the obvious disadvantage of using a radioactive tracer in vivo. Therefore, we developed a stable isotope dilution method to quantify Rₐ oxalate. In the present chapter this method is described.

MATERIALS AND METHODS

Study subjects

Three adult patients with PH1 were included in this study. Patients 2 and 3 are siblings. AGT deficiency was established either by measurement of AGT activity in a liver biopsy specimen (19) or by mutation analysis of the AGXT gene (7). The patients were compared with three healthy adults. Renal function as assessed by GFR estimated from serum creatinine (20) was lower in patients with PH1 than in control subjects, but still above the threshold at which systemic oxalosis progressively occurs (6). Patient 1 was pyridoxine responsive, whereas patients 2 and 3 were not. Patient 2 was treated with hyperhydration and supportive medication (magnesiumhydroxide and dihydrotachysterol), whereas patient 3 was only treated with supportive medication.

All studies were approved by the Institutional Review Board. All subjects gave informed consent prior to the studies.

Study protocol

Three days prior to the study all subjects maintained a low-oxalate diet to eliminate dietary influences during studies. All subjects started fasting 12 hrs before the start of the study and remained fasted throughout the whole study. They were allowed to drink water ad libitum. Two intravenous catheters were inserted into an antecubital vein of each arm. One catheter was used for administration of [1,2-¹³C]sodium-oxalate (99% pure; Cambridge Isotope Laboratories, Cambridge, MA) and the other for blood sampling. After collection of a baseline blood sample to determine the background enrichment of [1,2-¹³C]oxalate in plasma, a primed continuous infusion of [1,2-¹³C]sodium-oxalate was started. The priming bolus of [1,2-¹³C]sodium-oxalate was 0.11 μmol/kg in patients and 0.011 μmol/kg in control subjects, and the continuous infusion rate of [1,2-¹³C]sodium-oxalate was 1.25 nmol/kg-min in patients and 0.125 nmol/kg-min in control subjects. These infusion protocols were designed in order to reach a plasma [1,2-¹³C]oxalate enrichment of approximately 5%, based on previously published data on urinary oxalate excretion (15, 17, 21). Infusion of [1,2-¹³C]sodium-oxalate was continued for 8 hrs in all subjects.

Every hour a blood sample was drawn in order to determine
TABLE 07.1 Subject characteristics

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Sex</th>
<th>Age</th>
<th>Height</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>M</td>
<td>28.2</td>
<td>1.82</td>
<td>67.6</td>
</tr>
<tr>
<td>Patient 2</td>
<td>F</td>
<td>20.0</td>
<td>1.65</td>
<td>52.7</td>
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<tr>
<td>Patient 3</td>
<td>F</td>
<td>23.2</td>
<td>1.78</td>
<td>70.6</td>
</tr>
<tr>
<td>Control 1</td>
<td>M</td>
<td>30.6</td>
<td>1.79</td>
<td>63.0</td>
</tr>
<tr>
<td>Control 2</td>
<td>M</td>
<td>30.6</td>
<td>1.94</td>
<td>91.2</td>
</tr>
<tr>
<td>Control 3</td>
<td>F</td>
<td>22.3</td>
<td>1.75</td>
<td>68.0</td>
</tr>
</tbody>
</table>

[1,2-\textsuperscript{13}C]oxalate enrichment in plasma. Blood samples were collected in heparin Microtainers (Becton–Dickinson, Franklin Lakes, NJ, USA), which were immediately centrifuged at 3000 rpm for 10 min. Exactly 1 ml of plasma was transferred into a glass tube containing 0.4 g sodium chloride (in quadruple). Plasma was acidified with 37% hydrochloric acid in order to precipitate protein and in order to eliminate any possible oxalate formation from ascorbate (22). Hereafter, the acidified plasma was stored at -20\degree C until analysis.

Prior to initiation of [1,2-\textsuperscript{13}C]sodium-oxalate infusion all subjects were asked to void in order to empty their bladder. Hereafter, all urine was collected from the subjects until the end of [1,2-\textsuperscript{13}C]sodium-oxalate infusion in order to calculate urinary oxalate excretion during studies. Urine was acidified immediately after collection and was stored at -20\degree C until analysis.

**Analytical methods**

Plasma oxalate concentration and [1,2-\textsuperscript{13}C]oxalate enrichment:

Plasma oxalate concentration and [1,2-\textsuperscript{13}C]oxalate enrichment were determined in separate runs. Briefly, the acidified plasma was extracted twice with 4 ml ethylacetate and dried under a stream of nitrogen. The O-t-butyldimethylsilyl-quinoxalinol derivative of oxalate was formed with o-phenylenediamine and MTBSTFA/pyridine. An aliquot was injected into a GC/MS system (HP 6890 series GC system and 5973 Mass Selective Detector, Agilent Technologies, Palo Alto, CA, USA). Separation was achieved on a CP-SIL 19CB capillary column (30 m x 0.25 mm x 0.15 μm, Varian, Middelburg, The Netherlands). Selected ion monitoring (EI), data acquisition and quantitative calculations were performed using the Agilent Technologies Chemstation software. The oxalate derivative was monitored at m/z 333 for oxalate and m/z 335
for [1,2-13C]oxalate. For quantification of the enrichment a standard curve with known enrichments of [1,2-13C]oxalate was derivatized as described before. Oxalate tracer/tracee ratio's were calculated as described by B.W. Patterson et al (24).

In order to measure plasma oxalate concentration, [1,2-13C]oxalate was added to the acidified plasma as an internal standard. A correction was made for the contribution of the tracer to the internal standard peak. Oxalate concentration in the plasma samples was then calculated from a standard curve with known concentrations of oxalate.

**Urinary oxalate and creatinine concentration:**

Urinary oxalate concentration was determined by ion chromatography (Dionex-DX 300) (25). Urinary creatinine concentration was determined using spectrophotometry according to the Jaffé method.

**Calculations**

**Rates of oxalate appearance (Ra oxalate) and disappearance (Rd oxalate):**

Ra oxalate was calculated with Steele's steady-state equation (26). In the presence of isotopic steady-state Ra oxalate equals Rd oxalate and therefore also reflects the rate of oxalate clearance from plasma.

**Rate of urinary oxalate and creatinine excretion:** the rate of urinary oxalate excretion (μmol/kg-min) was calculated by multiplying urinary oxalate concentration (μmol/L) by total urinary volume (L), which was then divided by the collection time (min) and by bodyweight (kg) in order to calculate the rate of urinary oxalate excretion in all subjects.

The rate of urinary creatinine excretion (μmol/kg-min) was calculated in the same way in all subjects.

<table>
<thead>
<tr>
<th>Enzyme activity (normal range)*</th>
<th>Mutation analysis‡</th>
<th>Plasma creatinine</th>
<th>GFR †</th>
</tr>
</thead>
<tbody>
<tr>
<td>nmol/min-mg protein</td>
<td>allele 1/allele 2</td>
<td>μmol/L 1.73 m2</td>
<td>ml/min</td>
</tr>
<tr>
<td>9.8 (135 – 179)</td>
<td>ND</td>
<td>74</td>
<td>102.0</td>
</tr>
<tr>
<td>ND</td>
<td>c.33_34insC / c.33_34insC</td>
<td>107</td>
<td>63.1</td>
</tr>
<tr>
<td>ND</td>
<td>c.33_34insC / c.33_34insC</td>
<td>83</td>
<td>68.2</td>
</tr>
<tr>
<td>ND</td>
<td>ND</td>
<td>56</td>
<td>144.5</td>
</tr>
<tr>
<td>ND</td>
<td>ND</td>
<td>53</td>
<td>123.</td>
</tr>
<tr>
<td>ND</td>
<td>ND</td>
<td>58</td>
<td>106.9</td>
</tr>
</tbody>
</table>

† van Woerden et al Kidney Int 2004;66(2): 746-752
‡ estimated from serum creatinine; Levey et al Clin Chem 2007;53(4): 766-772

ND = not determined
RESULTS

Plasma enrichment of [1,2-13C]oxalate expressed as TTR (%) in all subjects is shown in FIGURE 07.1. An increase in TTR during studies was observed in all three patients until approximately 360 min of [1,2-13C]sodium-oxalate infusion. Hereafter, TTR reached a plateau in all three patients. In the control subjects, TTR remained constant after 120 min of [1,2-13C]sodium-oxalate infusion.

In all subjects, $R_a$ oxalate was calculated using the mean TTR in the blood samples at $t=360$, $t=420$ and $t=480$ min. $R_a$ oxalate was 0.031, 0.029 and 0.024 μmol/kg·min in patients 1, 2 and 3, respectively. In control subjects 1, 2 and 3 $R_a$ oxalate was 0.015, 0.017 and 0.016 μmol/kg·min, respectively.

Median (range) plasma oxalate concentration at isotopic equilibrium was 8.6 (8.6-9.6), 11.3 (11.1-11.8) and 10.1 (9.8-10.7) μM in patients 1, 2 and 3, and 3.2 (2.6-4.1), 3.7 (3.3-5.1) and 3.7 (3.4-4.2) μM in control subjects 1, 2 and 3, respectively.

Urinary oxalate excretion during [1,2-13C]sodium-oxalate infusion was 0.035, 0.031 and 0.035 μmol/kg·min in patients 1, 2 and 3, and 0.002, 0.005 and 0.004 μmol/kg·min in control subjects 1, 2 and 3, respectively. Urinary creatinine excretion during [1,2-13C]sodium-oxalate infusion was 0.15, 0.16 and 0.14 μmol/kg·min in patients 1, 2 and 3, and 0.14, 0.15 and 0.13 μmol/kg·min in control subjects 1, 2 and 3, respectively.

DISCUSSION

In this paper a novel stable isotope dilution method is described in order to quantify oxalate kinetics in plasma. This method may be used as a parameter to evaluate the efficacy of therapeutic interventions in patients with PH1 and renal insufficiency, since plasma oxalate concentration and urinary oxalate excretion are not reliable parameters for...
endogenous oxalate production (EOP) in these patients. We used a primed continuous infusion of [1,2-\(^{13}\)C]sodium-oxalate in order to quantify \( R_a \) oxalate in both patients with PH1 and age matched healthy control subjects. Renal function was comparable in all subjects as their urinary creatinine excretion rate was approximately similar. Previously, a single injection stable isotope method with [1,2-\(^{13}\)C]sodium-oxalate has been described to quantify oxalate turn-over (27). However, single injection \([^{14}\text{C}]\)oxalate methods have been shown to overestimate oxalate turn-over when compared to constant \([^{14}\text{C}]\)oxalate infusion methods as with the former a non-negligible amount of tracer will already be excreted in urine prior to homogenous distribution of the tracer throughout the pool, causing an erroneously high calculated rate of oxalate turn-over (15). In addition, numerous blood samples are generally required to accurately calculate substrate turn-over in plasma from the decay in isotopic enrichment of single injection of a substrate tracer. Therefore, we choose to device a primed continuous oxalate tracer method as this would be a more clinically applicable method to quantify \( R_a \) oxalate.

After approximately 360 min of a primed continuous infusion of [1,2-\(^{13}\)C]sodium-oxalate a steady-state in plasma isotopic enrichment was reached in the patients with PH1. In control subjects, a steady-state in isotopic enrichment was already present after only 120 min of [1,2-\(^{13}\)C]sodium-oxalate infusion. The latter is consistent with a previous study using a primed continuous infusion of \([^{14}\text{C}]\)oxalate in healthy subjects (15). This difference in equilibration period between patients and control subjects is caused by the higher metabolic pool of oxalate (the product of plasma oxalate concentration and of oxalate distribution volume) in patients with PH1 as compared to healthy subjects (6,17,18), as a steady-state in plasma [1,2-\(^{13}\)C]-oxalate enrichment can only be achieved when the oxalate tracer has been evenly dispersed throughout the whole metabolic pool. This also implicates that a longer equilibration period or a higher priming bolus of [1,2-\(^{13}\)C]sodium-oxalate is needed in patients with PH1 and renal insufficiency.

\( R_a \) oxalate calculated at isotopic equilibrium was in the same range in all three patients and closely resembled urinary oxalate excretion. Since GFR in the patients was above the threshold at which systemic oxalosis is suggested to occur (6), and almost all oxalate is cleared from plasma by renal clearance (28), \( R_a \) oxalate was expected to equal urinary oxalate excretion. This is indeed reflected by our data. Furthermore, our data suggest that daily oxalate production in patients with PH1 would amount to 2.55 mmol per day. This is in concordance with a previous estimate of daily oxalate production in patients with PH1 (15). Finally, although a high variation in plasma oxalate concentration has been reported in the patients with PH1, plasma oxalate concentration in our patients as quantified with our method was consistent with reported data on plasma oxalate concentration in patients with PH1.
with a preserved renal function (6,15,17). Therefore, \( R_a \) oxalate may be accurately quantified in patients with PH1 after a primed continuous infusion of \([1,2-13C]\)sodium-oxalate lasting at least 360 min as described in the present chapter.

In healthy control subjects, however, \( R_a \) oxalate was higher than expected as it exceeded urinary oxalate excretion several fold. Daily oxalate production in healthy subjects would amount to 1.72 mmol per day based on the \( R_a \) oxalate data and to 0.41 mmol per day based on the urinary excretion data. The latter is in good agreement with previous data on daily urinary oxalate excretion in control subjects (15,17, 28). This suggests that \( R_a \) oxalate as determined by our method is overestimated. Furthermore, although a large variation has been reported in plasma oxalate concentration in healthy subjects, as summarized in a recent study describing a stable isotope dilution method to quantify plasma oxalate and glycolate concentration \textit{in vitro} (29), plasma oxalate concentration as determined in our control subjects was in the higher reported range. The same dilemma was encountered in the study of France \textit{et al} who described a single injection \([1,2-13C]\)sodium-oxalate method to quantify \( R_a \) oxalate and found a daily oxalate production rate of 1.89 mmol in a healthy subject (27). These authors contributed this high production rate of oxalate to \textit{in vitro} oxalogenesis from ascorbate despite acidification of the plasma samples after collection. We cannot rule out that some \textit{in vitro} oxalogenesis from ascorbate occurred before analysis of the samples as this has been shown to occur in acidified frozen urine during prolonged storage (23). If so, this will have caused an overestimation of plasma oxalate concentration and of \( R_a \) oxalate in control subjects. Alternatively, urinary oxalate excretion could have been underestimated in control subjects due to precipitation of oxalate with calcium \textit{in vitro}. This seems less likely, however, as urinary super saturation is low in control subjects (30) and urinary acidification has been shown effective in preventing calcium-oxalate precipitation (23). Since both \( R_a \) oxalate and plasma oxalate concentration are higher in patients with PH1, the relative contribution of \textit{in vitro} oxalogenesis to total oxalate concentration in the plasma samples and \( R_a \) oxalate is smaller in patients than in control subjects and is probably negligible in patients with renal insufficiency exhibiting a much higher plasma oxalate concentration. To limit the influence of possible \textit{in vitro} oxalogenesis on the determination of \( R_a \) oxalate and plasma oxalate concentration, plasma samples should be immediately acidified and analyzed as soon as possible after their collection.

In conclusion, the reported stable isotope method to quantify \( R_a \) oxalate seems accurate in patients with PH1, but not in control subjects. This method may be used to monitor therapeutic interventions in hyperoxaluric patients with renal insufficiency as plasma oxalate concentration and urinary oxalate excretion are inaccurate parameters of EOP in these patients.
REFERENCES


