Extraction of glyceric and glycolic acids from urine with tetrahydrofuran: utility in detection of primary hyperoxaluria

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Primary hyperoxaluria (PH) is an autosomal recessive metabolic abnormality characterized by excessive oxalate excretion leading to nephrocalcinosis and progressive renal dysfunction. Type I primary hyperoxaluria (PH I) results from a deficiency of alanine:glyoxylate aminotransferase, whereas type II disease has been traced to a deficiency of D-glycerate dehydrogenase. The two syndromes are often distinguished on the basis of organic acids that are coexcreted with oxalate: glycolate and L-glycerate in type I and type II disease, respectively. Routine organic acid analysis with diethyl ether extraction followed by gas chromatographic analysis failed to detect normal and increased concentrations of these diagnostic metabolites. Subsequent extraction of urine with tetrahydrofuran (THF), however, extracted 75% of added glycerate, 42% of added glycolate, and 75% of added ethylphosphonic acid (internal calibrator). THF extraction was analytically sensitive enough to allow determination of normal excretion of glycolate (14–72 μg/mg creatinine) and glycerate (0–5 years, 12–177 μg/mg creatinine and >5 years, 19–115 μg/mg creatinine). Four of five patients with PH I and both patients with type II disease were correctly identified. Thus, THF extraction is a convenient adjunct to routine organic acid analysis and facilitates the detection of PH.

INDEXING TERMS: inborn errors of metabolism • oxalates • renal calculi • laboratory diagnosis • gas chromatography

Oxalate excreted in urine arises from metabolism of amino acids and carbohydrates in liver cytosol and peroxisomes [1, 2]. Oxalate is ordinarily a minor end product of these pathways. In peroxisomes, glyoxylate is derived by oxidation of glycine and glycolate, and then is largely transaminated to glycine through the action of alanine:glyoxylate aminotransferase (AGT, E.C. 2.6.1.44), but can alternatively be oxidized to oxalate via l-2-hydroxyacid oxidase (E.C. 1.1.3.1).7 In the cytosol, hydroxypyruvate derived from glucose and fructose is primarily reduced to l-glycerate or D-glycerate by lactate dehydrogenase (E.C. 1.1.1.27) or D-glycerate dehydrogenase (GDH, E.C. 1.1.1.29), respectively. Under normal circumstances a small amount of hydroxypyruvate is also oxidized to oxalate through pathways that remain undefined. Primary hyperoxaluria (PH) results from inherited deficiency of AGT or GDH, which ordinarily metabolize glyoxylate and hydroxypyruvate to less toxic products. The net effect of these deficiencies is an increased commitment of intermediates to oxalate, resulting in increased urinary excretion of oxalate, supersaturation of urine with calcium oxalate, and, in advanced disease, systemic deposition of calcium oxalate. Formation of renal stones and the resulting renal dysfunction are the presenting features of the disease [3, 4]. This disorder is distinct from enteric hyperoxaluria, which results from hyperabsorption of oxalate from the digestive tract [5].

Type I primary hyperoxaluria (PH I) is an autosomal

7 Nonstandard abbreviations: AGT, alanine:glyoxylate aminotransferase; GDH, D-glycerate dehydrogenase; PH, primary hyperoxaluria; PH I (II), primary hyperoxaluria type I (type II); GC-MS, gas chromatography–mass spectrometry; THF, tetrahydrofuran; and BSTFA, bis(trimethylsilyl)trifluoracetamide.
recessive deficiency of AGT with increased excretion of both oxalate and glycolate in urine [6–10]. Type II primary hyperoxaluria (PH II) results from a deficiency of GDH, leading to coaccumulation of oxalate and 1-glycerate in urine [11, 12]. European literature has reported that 1–2% of childhood cases of end-stage renal disease may be attributable to some form of PH [13, 14]. PH I is diagnosed more commonly, has an earlier onset, and a poorer prognosis than PH II [11, 15, 16]. Some cases of PH I are responsive to pyridoxine (an AGT cofactor), high fluid intake, phosphate treatment, or citrate administration, yet heroic measures such as kidney and (or) liver transplantation are commonly required after irreversible kidney damage [2, 17–20]. Patients with PH II have a much more benign prognosis, though some do progress to end-stage renal disease [12].

Thus, detection of PH and the distinction between PH I and PH II is necessary for patients who might benefit most from aggressive intervention. Currently this is accomplished by determination of AGT activity in liver biopsy specimens [6, 7] and measurement of glycolate and glycerate excretion. Liver biopsy allows direct assessment of enzymatic deficiency, but has several disadvantages: patient discomfort, the risk of an invasive procedure, cost, long turnaround time, and (currently) no source for GDH determination. In addition, individuals homozygous for AGT deficiency sometimes have enzyme activities indistinguishable from heterozygotes who are clinically normal [21, 22]. Reliable detection of 1-glycerate and glycolate in urine may obviate the need for biopsy in many cases. Specific methods for glycolate and glycerate determination have been developed [23, 24], but most laboratories, even specialized ones aimed at detecting genetic metabolic disease, do not maintain specific testing protocols for glycolate and 1-glycerate. These compounds are only occasionally detected in routine organic acid analysis by gas chromatography–mass spectrometry (GC-MS) because their polar character makes them difficult to extract from urine with diethyl ether or ethyl acetate, the common solvents used in routine organic acid analysis [25]. With current methods, normal concentrations of glycolate and glycerate are rarely detected; extreme increases in excretion may be required for detection. This could partly explain the observation by Danpure that 30% of PH I patients with marked hyperoxaluria did not display increased glycolate excretion [26]. We reasoned that a procedure that increased the efficiency of glycolate/glycerate extraction would provide more accurate normal ranges and potentially improve the sensitivity of organic acid analysis to detect and distinguish between PH I and PH II earlier in the course of the disease. Rimoldi et al. improved the extraction of polar compounds such as citric, hydroxybutyric, and orotic acids from urine by using tetrahydrofuran (THF) [27]. Here, we describe the utility of THF extraction in aiding the diagnosis of PH.

**Materials and Methods**

**Materials.** Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and oxalic, hippuric, succinic, and d,l-lactic acids were obtained from Sigma (St. Louis, MO). Ethylphosphonic acid, glycolic acid, and THF were purchased from Aldrich (Milwaukee, WI). d,l-Glyceric acid was obtained from ICN (Cleveland, OH).

**Specimens.** Random urine specimens were adjusted to pH 2 to ensure complete recovery of oxalate and maintained at −20 °C. A preliminary experiment showed that glycerate and glycolate were stable in acidified urine for up to 3 months. Oxalate salts precipitated in as little as 2 weeks of storage even at pH 2, and thus oxalate was not measured on specimens stored for longer periods. Normal ranges for glycerate, glycolate, and oxalate (normalized to creatinine) were established with 65 specimens from children and adults without evidence of liver or kidney disease. Specimens from children <6 months of age were obtained from outwardly healthy children visiting the outpatient clinic at St. Louis Children’s Hospital. One glycolate and seven oxalate values were statistically excluded from the normal range by the outlier analysis of Reed et al. [28]; oxalate:creatinine ratios are known to be nonnormally distributed in children [9]. This study was conducted in accord with a protocol approved by the human studies committees of Washington University and the Mayo Clinic.

**Analytical.** A volume of urine containing 500 µg of creatinine was diluted to 5 mL. Ethylphosphonic acid (250 µg) was added as internal calibrator. The urine was saturated with NaCl and acidified to pH 1 with HCl before extraction. Extraction was performed three times with 5 mL of diethyl ether and then five times with 5 mL of THF. THF and ether extracts were pooled separately and concentrated to dryness under nitrogen. Residual water was removed by reconstituting the residue with 200 µL of benzene to form an azeotropic mixture and again reducing it to dryness under nitrogen. Residues were then derivatized by incubation in pyridine:BSTFA (1:1 by vol) for 15 min at 60 °C. Calibrators were dissolved in pyridine and derivatized with an equal volume of BSTFA before use. Derivatives were analyzed on a Varian 3700 gas chromatograph (Varian Instrument Group, Palo Alto, CA) equipped with a DB-1 column (0.53 mm i.d.; J) Colbert Assoc., St. Louis, MO) by using a temperature program of 7 min at 80 °C followed by a rise to 260 °C at 6 °C per minute. The injector and detector temperatures were both 250 °C. Compounds were detected by flame ionization and identity of the peaks was confirmed with a Finnigan ITD mass spectrometer (Finnigan MAT, San Jose, CA). Quantification of oxalate, glycolate, and glycerate was based on the detector response to a known amount of each compound and corrected for recovery of the internal calibrator. Glycolic, oxalic, and ethylphosphonic acid calibrators were prepared by dissolving highly pure material...
in pyridine and derivatizing immediately before use. Glyceric acid was supplied as a syrup with a significant water content and was lyophilized before dissolving in pyridine. Creatinine was determined on the Vitros 700 XR (Johnson and Johnson, Rochester, NY).

**Results**

**Extraction of glycerate and glycolate.** An aliquot of urine containing 500 µg of creatinine and supplemented with 250 µg each of succinate (C₄H₆O₄), hippurate (C₉H₉O₃), oxalate (C₂H₄O₄), glycerate (C₃H₇O₄), and glycolate (C₂H₅O₃) was saturated with NaCl, acidified, and extracted three times with 5.0 mL of ether (standard protocol), then multiple times with 5.0 mL of THF. The first ether and THF extracts were concentrated, derivatized, and analyzed by GC. The more hydrophobic molecules, hippurate and succinate, were extracted effectively by ether while the very polar C-2 and C-3 acids were not extracted from the urine until THF was used (Fig. 1). Lactate, with intermediate hydrophobicity, was partially extracted with ether but predominantly recovered in THF extracts. Several other polar compounds (phosphate, urea, and citrate) were also efficiently extracted with THF. Ethylphosphonic acid (C₂H₇PO₃) was used as an internal calibrator because of its absence in human urine and polar character similar to glycerate and glycolate. It was extracted exclusively with THF.

**Optimization of THF extraction.** The goal of the protocol was to extract as much of each diagnostic compound as possible and to match the recovery of each to the internal calibrator. To determine the optimal number of extractions, 250 µg each of oxalate, glycerate, glycolate, and ethylphosphonate were added to an aliquot of normal urine (containing 500 µg creatinine) that was extracted three times with ether and then repeatedly extracted with THF. When individual THF extracts were analyzed, oxalate was recovered predominantly in two extractions, whereas five extractions were required to recover a comparable amount of glycerate, glycolate, and ethylphosphonate (Table 1). In subsequent studies, pooling of five successive extracts resulted in recovery of 43% ± 13%, 76% ± 7%, 43% ± 6%, and 71% ± 9% of oxalate, glycerate, glycolate, and ethylphosphonate, respectively (mean ± SD, n = 8). Correction of values based on the recovery of the internal calibrator, therefore, slightly underestimates the amount of glycolate and oxalate present. Extraction of glycolate and glycerate was linear up to 1000 µg/mg creatinine ($S_{\text{mix}} = 18$ µg/mg creatinine, $r = 0.9883$, and $S_{\text{mix}} = 47$ µg/mg creatinine, $r = 0.9683$, respectively). A precision study consisting of six runs over 6 weeks was performed with a normal urine pool containing mean glycolate and glycerate concentrations of 28.5 and 80.6 µg/mg creatinine, respectively. At these concentrations, where imprecision is likely to be high, CVs were 17% and 28.8% for glycolate and glycerate determination, respectively. Peaks corresponding to 5 µg/mg creatinine were readily detectable above the baseline signal.

**Analysis of patient specimens.** Normal ranges were determined from random urine specimens obtained from healthy children of laboratory employees and adults with no history of renal disease and are reported as the actual range of observed values. Specimens from infants <6 months of age were obtained from outwardly healthy children visiting the outpatient clinic at St. Louis Children’s Hospital. Glycerate excretion in this healthy population was dependent on age (Fig. 2). Children <5 years of age (n = 19) displayed higher excretion rates of glycerate (12–177 vs 19–115 µg/mg creatinine) than older
children and adults \( (n = 39) \). Normal glycolate excretion was 14–72 µg/mg creatinine \( (n = 64) \). No gender differences were apparent.

The new extraction protocol was applied to specimens from 16 PH patients seen at the Mayo Clinic Division of Nephrology to assess the ability of improved glycolate/glycerate extraction to discriminate between PH I and PH II. Patients were all 5 years of age or older and were classified by: \( (a) \) prior history of renal dysfunction, \( (b) \) hyperexcretion of oxalate/glycerate or oxalate/glycolate (determined by standard organic acid analysis at reference laboratories), \( (c) \) liver AGT activity in liver biopsy material (when performed), and \( (d) \) response to pyridoxine. Pyridoxine (a cofactor for AGT but not GDH) augments existing AGT activity in some patients, resulting in normalized oxalate excretion, and is therefore a hallmark of PH I. We examined specimens from nine individuals whose urine oxalate excretion was responsive to pyridoxine. Consistent with clinical response, glycolate and glycerate concentrations were within normal limits in this population (data not shown). Five other patients were classified as PH I, three by history of marked hyperoxaluria and glycolate hyperexcretion (DM, LF, AJ), one as the result of AGT deficiency by liver biopsy (NB), and one other (AM) on the basis of glycolate hyperexcretion and biopsy-proven AGT deficiency in an affected sibling. Four of these five patient specimens had increased glycolate (Table 2). The initial specimen from AM displayed high-normal excretion of glycolate (53 µg/mg creatinine), but a subsequent specimen did show high glycolate concentration (78 µg/mg creatinine). Two PH II individuals were classified by a history of oxalate/glycerate hyperexcretion at a reference laboratory. Both patients had increases in glycerate that were at least threefold above the upper limit of normal with our new method. In summary, then, four of five PH I patients unresponsive to pyridoxine and both PH II patients were detected by THF extraction.

**Discussion**

Our results show that THF extraction of urine (as an adjunct to routine organic acid analysis) significantly improves sensitivity for polar compounds such as glycolate and glycerate and allows even normal excretion to be quantified. The ability to detect normal concentrations of these compounds may improve the utility of the test in discriminating between normal and affected individuals. The efficiency of extraction of glycolate and glycerate, though much greater with THF than other standard solvents, is still incomplete, a fact that decreases precision of analysis. Nevertheless, our data indicate that precision and accuracy with THF extraction are sufficient to readily distinguish PH from normality. Performed after ether extraction, THF extraction requires no additional equipment and little additional effort in laboratories already performing organic acid analysis.

The THF extraction strategy presented here compares favorably with other methods involving direct (no extraction) determination [29] or alternative extraction techniques (anion-exchange chromatography) to detect glycolate [30]. Reported normal glycerate values, however, vary widely [12, 31]. These differences are possibly due to the range of methods used for quantifying glycerate and

**Table 1. Recovery of oxalate, glycerate, glycolate, and ethylphosphonate in five successive THF extracts.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>1st</th>
<th>2nd</th>
<th>3rd</th>
<th>4th</th>
<th>5th</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxalate</td>
<td>48</td>
<td>14</td>
<td>2.8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glycerate</td>
<td>15</td>
<td>18</td>
<td>16</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>Glycolate</td>
<td>11</td>
<td>10</td>
<td>9.2</td>
<td>7.4</td>
<td>4.7</td>
</tr>
<tr>
<td>Ethylphosphonate</td>
<td>26</td>
<td>23</td>
<td>14</td>
<td>8.6</td>
<td>5.0</td>
</tr>
</tbody>
</table>

A representative urine specimen containing 250 µg of each of the indicated compounds was extracted five successive times with THF. Extracts were concentrated, derivatized, and analyzed separately.
their standardization. Standardization is particularly difficult with GC-MS assays. Glyceric acid is available commercially as a syrup with significant water content or as its calcium salt, which is insoluble in organic solvents and in the derivatizing agents used. Both forms must be manipulated further to provide a free acid form suitable for standardization. Differences due to standardization do not affect the ratio of increased to normal concentrations of glycerate and would, therefore, not diminish the ability of THF extraction to identify those patients with increased excretion rates of glyceric acid.

Interpretation of results must be made with some qualification. First, the technique described here does not distinguish between the d and l forms of glyceric acid. Further investigation is required to confirm excretion of the l isomer, which is specific to PH II. Second, excretion of glycerate and glycolate will likely vary considerably within individuals. For instance, one sample in our study contained high-normal concentrations of glycolate; a second sample was clearly increased. Finally, progressive glomerular damage reduces filtration of glycerate and glycolate, so interpretation must always take into account the extent of residual renal function. With these considerations in mind, THF extraction of glycerate and glycolate as an adjunct to routine organic acid analysis should enable early detection and distinction of PH and avoid the need for liver biopsies in many patients.

References


Table 2. Application of THF extraction to specimens from patients with PH I or PH II.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Clinical status</th>
<th>Glycolate</th>
<th>Glycerate</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB</td>
<td>PH I</td>
<td>191 µg/mg creatinine, 55.4 µg/mL</td>
<td>31 µg/mg creatinine, 9.1 µg/mL</td>
</tr>
<tr>
<td>DM</td>
<td>PH I</td>
<td>165 µg/mg creatinine, 67.7 µg/mL</td>
<td>30 µg/mg creatinine, 12.3 µg/mL</td>
</tr>
<tr>
<td>LF</td>
<td>PH I</td>
<td>92 µg/mg creatinine, 4.5 µg/mL</td>
<td>&lt;5 µg/mg creatinine, &lt;0.2 µg/mL</td>
</tr>
<tr>
<td>AJ</td>
<td>PH I</td>
<td>281 µg/mg creatinine, 117.7 µg/mL</td>
<td>71 µg/mg creatinine, 29.7 µg/mL</td>
</tr>
<tr>
<td>AM</td>
<td>PH I</td>
<td>53 µg/mg creatinine, 19.9 µg/mL</td>
<td>32 µg/mg creatinine, 12.0 µg/mL</td>
</tr>
<tr>
<td>RD</td>
<td>PH II</td>
<td>18 µg/mg creatinine, 1.3 µg/mL</td>
<td>314 µg/mg creatinine, 22.3 µg/mL</td>
</tr>
<tr>
<td>GD</td>
<td>PH II</td>
<td>23 µg/mg creatinine, 11.5 µg/mL</td>
<td>1359 µg/mg creatinine, 680.9 µg/mL</td>
</tr>
<tr>
<td>Normals</td>
<td></td>
<td>14–72 µg/mg creatinine, 19–115 µg/mL</td>
<td></td>
</tr>
</tbody>
</table>

Random urine specimens were obtained at visits to the Mayo Nephrology Clinic. All patients were 5 years of age or older. The normal range associated with this population (age ≥5 years) is given in the last row of the table. (To convert µg/mL glycolate and µg/mL glycerate to µmol/L, multiply by 12.99 and 9.35, respectively.)


