Quantification of Orotic Acid in Dried Filter-Paper Urine Samples by Stable Isotope Dilution

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A rapid, sensitive, and specific method for quantification of orotic acid from dried filter-paper urine samples is described. The method involves stable isotope dilution with 1,3-$^{15}$N$_2$-orotic acid and analysis by gas chromatography–mass spectrometry. The assay is sufficiently sensitive to be used with solvent extraction techniques commonly used for urinary organic acid analysis. Extraction efficiencies of both native and isotopic orotic acid from dried filter paper and from water were 31% and 28%, respectively. The concentration of orotic acid in dried filter-paper urine specimens from 50 healthy controls was 1.1 ± 0.67 (mean ± SD) mmol/mol of urinary creatinine. The same 50 urine samples, analyzed directly from a 5-mL aliquot of liquid urine, gave values of 0.93 ± 0.51. The correlation coefficient between the results obtained by the two different collection methods was 0.87. Age-related reference values in filter-paper samples are also reported. The concentrations, which are normalized to urinary creatinine, decrease with age. This method is applicable to rapid screening for urea cycle disorders and may also be used for carrier testing of ornithine transcarbamylase deficiency.

**Indexing Terms:** gas chromatography–mass spectrometry/hyperammonemia/urea cycle/screening methods/ornithine transcarbamylase/inherited disorders/pyrimidines

Orotic acid (Fig. 1) is an intermediary metabolite of the de novo pyrimidine synthesis pathway, deriving from carboxylation of aspartic acid. Patients with hyperammonemia, caused by deficiencies in various urea cycle enzymes, often show increased urinary excretion of orotic acid. The disorder most associated with orotic aciduria is ornithine transcarbamylase (OTC; EC 2.1.3.3) deficiency. In addition, patients with other disorders associated with the urea cycle, such as arginemia, citrullinemia, argininosuccinic aciduria, lysinuric protein intolerance, and hyperornithinemia, hyperammonemia, and homocitrullinuria, also exhibit orotic aciduria (1–3). Thus, orotic acid determination is an important test when attempting to narrow the differential diagnosis of hyperammonemia.

Normal human urine contains, trace amounts of orotic acid (4). The early colorimetric methods for orotic acid determination are simple and rapid, yet nonselective, which may give rise to false-positive results (5). Three previous reports describe the use of gas chromatography–mass spectrometry (GC-MS) to measure orotic acid, yet do not offer a wide range of normal controls and do use liquid urine samples (6–8). Although the frequency of urea cycle disorders is low, the rapid, automated, and accurate analysis of urine orotic acid with a very small and easily transportable sample is clinically important and very useful. Collection of liquid urine, especially from newborns, is difficult and time-consuming, and as a result is expensive. Moreover, shipment of frozen urine on frozen CO$_2$ is expensive and could be delayed, especially by air couriers. To circumvent these problems and to provide patients and clinicians with accurate and expeditious results, we have built upon our experience with GC-MS screening of filter-paper urine samples for neuroblastoma and organic aciduria and have developed a method for measuring orotic acid from identical samples. The method we report has the advantages of easy sample collection, easy shipping or delivery, and rapid analysis. Moreover, this method of collection and analysis can be used to measure orotic acid after allopurinol ingestion, which is used as a challenge test to uncover defective OTC enzyme function.

**Materials and Methods**

Orotic acid was purchased from Sigma Chemical Co., St. Louis, MO. 1,3-$^{15}$N$_2$-Orotic acid was purchased from Cambridge Isotope Labs., Cambridge, MA. Ethyl acetate was purchased from EM Industries, Gibbstown, NJ. \(N,O\)-Bis(trimethylsilyl)trifluoroacetamide containing 100 mL/L trimethylchlorosilane (BSTFA/TMCS) (Regisil RC-3) was purchased from Regis Chemical Co., Morton Grove, IL.

Liquid urine samples and aliquots of these samples dried on filter paper from 50 individuals, newborn to age 58 years, not affected by urea-cycle disorders were used to establish reference values for urine orotic acid concentrations and to compare the results obtained from both collection methods. These samples were referred to our laboratory for other urine tests.

Urine (5 mL) was poured onto 10 × 10 cm absorbant filter paper (no. 903; Schleicher & Schuell, Keene, NH), which was dried in room air. (When less urine is available, a proportionally smaller paper can be used.) A 20-cm$^2$ section was cut from the paper, fan-folded, and placed in a disposable 16 × 125 mm polypropylene tube. The soluble urine components were eluted with 5
mL of water by mechanical shaking for 5 min and the eluate was then processed as described below for liquid urine.

Liquid urine was handled as follows: A 5-mL aliquot of random urine was put into a disposable 16 × 125 mm polypropylene tube. A 100-μL aliquot was removed for urinary creatinine determination by Folin's method with the Jaffé reaction (9). 1,3-[15N2]Orotic acid (0.64 μmol (100 μg)) was added to the urine or dried urine filter paper as the internal standard. The sample was saturated with 1 g of sodium chloride and acidified to pH <1 with 0.2 mL of 5 mol/L HCl. The organic acids were extracted twice with 6 mL of ethyl acetate for 5 min by using a mechanical reciprocal shaker. The organic layers were removed and dried under a stream of nitrogen at 50°C. Derivatization to form trimethylsilyl (TMS) derivatives of the organic acids was accomplished with 100 μL of BSTFA/TMCS (10:1 by vol) solution for 20 min at 60°C, and the derivatized urine residue was then transferred into disposable injection vials. One microliter of the derivatized organic acid extract was injected into the gas chromatograph (model 5890 II; Hewlett-Packard, Avondale, PA) by an automated injection system. The acids were separated on a cross-linked 0.2 mm (o.d.) × 30 m 5% phenylmethylsilicone capillary column with a film thickness of 0.52 μm (Ultra 2; Hewlett-Packard).

The oven temperature program was started at 100°C and increased by 10°C/min up to 270°C, where it was held for 5 min, during which time the data were processed and the results reported by computer. Injection port and transfer line temperatures were 250° and 280°C, respectively. Split injection mode was used with a split ratio of 1:40 (lower split ratio results in more frequent ion source contamination, thus requiring more frequent maintenance). Flow rate of the helium carrier gas was ~1 mL/min and its linear velocity 35 cm/s.

Detection was by mass selective detector (5971 quadrupole MSD; Hewlett-Packard) equipped with an electron impact ion source that was "autotuned" and run in the high-resolution mode with selective-ion monitoring. The following ions were monitored within 1-min time windows: m/z 254 and 357 for natural orotic acid-triTMS, m/z 256 and 359 for 1,3-[15N2]orotic acid-triTMS (internal standard). Dwell time for each ion was 10 ms and the resolution was 0.5 atomic mass units. Quantification was performed with standard curves of m/z 254/256 and m/z 357/359 ion ratios, representing the ratio between various known amounts of natural orotic acid and 0.64 μmol (100 μg) of 1,3-[15N2]orotic acid. The orotic acid concentrations were normalized to the urinary creatinine concentration.

To test the stability of orotic acid on dried paper, we supplemented five aliquots of one urine sample with 0.13 μmol (20 μg) of natural orotic acid, applied this to filter paper, stored in room air, then subsequently analyzed at equal intervals within 2 weeks. To test the reproducibility of the method of collection and analysis, we analyzed separately five sections of one filter paper. Day-to-day variations of the assay were determined by daily analysis of two urine samples for 10 consecutive working days. To test this assay with positive controls, we assayed samples from two male patients and one female patient with OTC deficiency and compared their results with those of normal controls.

Results

Figure 2 shows the electron impact mass spectra for the natural and isotopic orotic acids and a sample of a selected ion tracing obtained from a filter-paper urine sample containing normal amounts of orotic acid. The prominent m/z 254 and 256 ions of the respective compounds were selected for monitoring and calculation of results. Comparing ion abundance from equal amounts of natural and isotopic orotic acid showed a 12% contribution ("spillover") of m/z 256 from the natural orotic acid and a 5% contribution of m/z 254 from the stable isotope. These spillovers are of minor significance because we use a relatively large amount of stable isotope, and the calibration curve, at least up to 25 μg, is linear. In cases of greatly increased orotic acid concentrations in ranges beyond the calibration curve, spillover may result in small errors in calculations that would not be clinically significant. Detector responses reflected as m/z 254/256 area ratios obtained from a constant amount (0.64 μmol, 100 μg) of 1,3-[15N2]orotic acid and various amounts of natural orotic acid (0–0.16 μmol, 0–25 μg) added to water and analyzed as described above showed a linear relation.

The equation for the response curve was y = 0.012x + 0.005 (r2 = 0.99). We also obtained results from the ratios of the less abundant ions m/z 357/359; both calculations agreed within ± 5%. The lower limit of detection calculated from blank runs (mean ± 3 SD, n = 10) is 1.09 μmol/L (0.17 mg/L). The lower limit of measurement with this method will vary with the urine concentrations but as little as 0.05 mmol/mol creatinine can be measured.

The extraction efficiency of orotic acid from liquid urine and urine absorbed onto filter paper supplemented with 0.64 μmol (100 μg) of orotic acid was consistently 25–30%, with no difference between liquid urine and filter-paper samples.

Dried filter-paper urine samples supplemented with 0.13 μmol (20 μg) of orotic acid and stored as long as 2 weeks at room temperature before analysis gave results of 0.12 ± 0.003 μmol (18.7 ± 0.48 μg, mean ± SD). Analysis of five 20-cm² sections of the same filter-paper urine specimen gave concentrations of 0.93 ± 0.04 mmol/mol creatinine (CV = 0.05).

Two control urine samples analyzed for 10 consecu-
tive working days gave results of 0.48 ± 0.03 and 0.13 ± 0.01 mmol/mol creatinine, respectively (CV = 0.07 and 0.05, respectively).

Figure 3 illustrates the comparison of orotic acid concentrations between liquid urine and dried filter-paper urine specimens of 50 control individuals. The mean ± SD in 50 control specimens analyzed in filter paper was 1.1 ± 0.61 mmol/mol creatinine, with a standard error of ± 0.09. The same 50 urine samples analyzed directly from a 5-mL aliquot of liquid urine gave a value of 0.93 ± 0.51, with a standard error of ± 0.07.

Table 1 shows age-related urinary orotic acid concentrations as measured from dried filter-paper urine specimens. The concentrations of orotic acid of three children with OTC deficiency, ages 1 month–6 years, were markedly increased over the normal range, and ranged from 167 to 360 mmol/mol creatinine.

Fig. 2. Mass spectra of natural orotic acid tri-TMS (A); 1.3-[15N2]orotic acid tri-TMS (B); and a selected-ion chromatogram of orotic acid analysis obtained from normal urine collected on filter paper (C).

Fig. 3. Correlation of orotic acid concentrations (mmol/mol creatinine) in dried filter-paper urine specimens and in liquid urine specimens obtained from 50 control individuals. The regression equation is \( y = 0.786x + 0.092, r^2 = 0.87 \); standard error about the regression is 0.06; and standard error of the intercept is 0.07.
Discussion

Collection of urine on filter paper followed by color "spot tests" for metabolic disorders and neural crest tumors has been applied before (10, 11). This collection method was used for urine metabolic screening programs in newborns in Massachusetts and Quebec, Canada (12, 13) and has also been applied in screening programs for neuroblastoma (14). If a method includes samples stored and shipped on dry paper, the cost for collection, storage, and shipment is substantially reduced. Other methods for measuring orotic acid have involved spectrophotometry and HPLC. The spectrophotometric method lacks specificity and may therefore give falsely positive results (5). HPLC methods, although reliable, do not have the specificity of detection that GC-MS detection offers. Reversed-phase HPLC has a shortcoming of early elution; therefore, on occasion, orotic acid may not be separable from other early-eluting compounds (15). The observation that the method described here yielded lower normal values than reversed-phased HPLC analysis supports the notion that GC-MS analysis of orotic acid by stable isotope dilution is more specific than HPLC and therefore more accurate. The ion-exchange HPLC method does not have the problem of early eluting compounds, but rather of orotic acid eluting late in the chromatographic analysis (16). Therefore, ion-exchange HPLC is time-consuming, hindering the analysis of a large number of samples by lacking a rapid turnaround time of results. GC-MS without stable isotope dilution has been used for orotic acid determination (8); however, without a suitable carrier (stable isotope), the low extraction efficiency means that a fraction of the small amounts of orotic acid present in urine will be lost during the analysis, causing inaccurate results. The above disadvantages are addressed by the current method, which is very sensitive, specific, and rapid.

Stable isotope dilution GC-MS analysis is well-suited for the quantification of orotic acid because it overcomes the problem of low extraction efficiency of this acid by using standard extraction methods for organic acid analysis. Whereas orotic acid is barely detectable by solvent extraction and full-scan GC-MS analysis, the stable isotope dilution method allows for much greater sensitivity by providing an ideal internal standard that also acts as a carrier, thus minimizing loss of the natural compound during the extraction and chromatographic analysis. Specificity is also greatly improved by the use of selected-ion monitoring and the highly reproducible retention times of the capillary column. As evidenced by the results, orotic acid is quite stable in the filter paper for enough time to allow shipment to a reference laboratory and analysis. In addition, the reproducibility of the method is very good, with a CV of 0.05 in multiple analyses of the same filter-paper sample.

We have previously applied selected-ion monitoring GC-MS to analyze filter-paper urine samples for rapid screening for neuroblastoma and organic aciduria (17, 18). These two tests measure organic acids after solvent extraction and could be performed simultaneously on the same sample in which orotic acid is analyzed. The test we describe thus provides an additional capability of measuring orotic acid on the same specimen, allowing a rapid screen for some of the urea cycle disorders in addition to screening for organic acidemias and neuroblastoma. We have shown previously that normalization to urinary creatinine is at least as good as relying on 24-h urine collection because the latter is frequently incomplete and expensive. The disadvantage of variability in random urine samples can easily be addressed by repeating a random sample rather than struggling with 24-h collection, which is very difficult in the pediatric population. Moreover, ease of collection and delivery is an important factor when biological samples are sent to reference laboratories for analysis.

Normal urine orotic acid concentrations normalized for creatinine concentrations decrease with age. These results were expected, having been seen also with other urine metabolites such as homovanillic acid and vanillylmandelic acid. The likely explanation for this is the increase in creatinine excretion in older individuals due to increased muscle mass compared with the smaller increase in excretion of other metabolites.

In summary, urinary orotic acid can be reliably determined from urine dried onto filter paper, alleviating the need for shipment of frozen urine. The method described is specific and accurate enough to be used for testing of carrier status for ornithine transcarbamylase deficiency on the basis of orotic acid excretion after administration of allopurinol (19).

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References
5. Harris ML, Oberholzer VG. Conditions affecting the colorim-

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Table 1. Age-related urinary orotic acid concentrations from dried filter-paper urine specimens of 50 control individuals analyzed by stable isotope dilution GC-MS.

<table>
<thead>
<tr>
<th>Age</th>
<th>n</th>
<th>Mean ± SD (range), mmol/mol urinary creatinine</th>
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<tbody>
<tr>
<td>0–1 years</td>
<td>16</td>
<td>1.31 ± 0.67 (0.12–3.07)</td>
</tr>
<tr>
<td>1–3 years</td>
<td>12</td>
<td>1.17 ± 0.62 (0.51–2.29)</td>
</tr>
<tr>
<td>3–9 years</td>
<td>12</td>
<td>0.97 ± 0.59 (0.40–2.32)</td>
</tr>
<tr>
<td>9–adult</td>
<td>10</td>
<td>0.65 ± 0.34 (0.20–1.20)</td>
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742 CLINICAL CHEMISTRY, Vol. 41, No. 5, 1995