Tiglylglycine Excreted in Urine in Disorders of Isoleucine Metabolism and the Respiratory Chain Measured by Stable Isotope Dilution GC-MS

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Tiglylglycine (TG), an intermediate product of the catabolism of isoleucine, is increased in the urine of patients with β-ketothiolase deficiency or with disorders of propionate metabolism. It is also implicated as a useful diagnostic marker in disorders of the respiratory chain. We present a method for the synthesis of TG and tiglyl-[13C,15N]glycine and the development of a stable isotope dilution mass spectrometric assay for TG. We compare data from controls with that from subjects with β-ketothiolase deficiency and propionyl-CoA carboxylase deficiency, and with six patients with enzyme-confirmed disorders of the respiratory chain. TG was increased in the urine of all of the patient groups. The increased TG excretion did not persist in one patient with a respiratory chain defect, which suggests that, in some patients, multiple sample analysis may be necessary to identify a respiratory chain defect. This is the first urinary compound to be implicated as a potential marker of disorders of the respiratory chain.

Indexing Terms: organic acids/reference range/mitochondrial enzyme defects/heritable disorders/Pearson syndrome

Tiglylglycine (TG) is a metabolic intermediate excreted in excess deficiencies of 2-methylacetoacetyl-CoA thiolase (2-MAA thiolase; EC 2.3.1.9) and propionyl-CoA carboxylase (PCC; EC 6.4.1.3) and in methylmalonic acidemia (I, 2). It is formed by the action of the mitochondrial enzyme glycine-N-acetyltransferase (EC 2.3.1.13) on accumulated tiglyl-CoA, an intermediate of isoleucine metabolism (3) (Fig. 1). Recently, we identified increased urinary TG in patients with disorders of complex I of the mitochondrial respiratory chain (4). It would be very useful to have a metabolic marker for complex I deficiency and other disorders of the respiratory chain, which are a very difficult group of disorders to diagnose. The only constant metabolic finding in most patients with respiratory chain disorders is a lactic acidemia that is metabolically indistinguishable from secondary lactic acidemia due to tissue anoxia (5).

The fact that TG is not commercially available has hindered the development of an accurate quantitative assay. Here, we report the chemical synthesis of TG and of stable-isotope-labeled tiglyl-[13C,15N]glycine ([13C,15N]TG), and detail their use in the development of a sensitive stable isotope dilution gas chromatographic–mass spectrometric (GC-MS) assay. We present data for urinary excretion of TG in controls, in subjects with various diseases including 2-MAA thiolase and PCC deficiencies, and in patients with enzyme-confirmed defects of the respiratory chain.

Materials and Methods

Synthesis of Labeled and Unlabeled TG

[13C,15N]Glycine (99 atom % pure) was obtained from Merck, Sharp and Dohme (Montreal, Canada). TG was synthesized by a modification of the procedure described by Rowley and Gerritsen (6). Tiglylchloride was prepared from tiglic acid according to Yamada (7) by reflux with thionyl chloride and distillation. Glycine (labeled or unlabeled, 10 mmol) was dissolved in 10 mL of 1 mol/L NaOH, and an equimolar amount of tiglylchloride was added slowly with stirring; the stirring was continued for 2 h more. The mixture was carefully acidified to pH 1 with 10 mol/L HCl with vortex-mixing, saturated with NaCl, and then extracted three times with ethyl acetate. The combined organic fractions were dried under a stream of nitrogen. Recrystallization was performed in ethyl acetate/hexane (R. I. Kelley, Johns Hopkins University, Baltimore, MD, personal communication). The purity of TG was >99% as judged by the lack of additional peaks on GC-MS and by the lack of glycine detection during amino acid analysis by ion-exchange chromatography. The molecular ion for [13C,15N]TG (see below) revealed >99% isotopic purity with no detectable molecular ion for TG; the yield of [13C,15N]TG was 27%. The melting range for each preparation (labeled and unlabeled) was 54–59°C.

Nuclear magnetic resonance (NMR) spectrometry of purified products. Proton-decoupled 13C NMR data were acquired on a Bruker (Billerica, MA) 200-MHz NMR spectrometer equipped with a TecMag acquisition computer and an Apple Macintosh Quadra 950 computer with MacNMR software. The compounds were analyzed as 100 g/L solutions in 5 mm NMR tubes and d<sub>6</sub>-dimethyl sulfoxide solvent. All signals were singlets unless otherwise noted (Fig. 2).
GC-MS of purified products. From a 1 mmol/L stock aqueous standard solution of TG and a 270 μmol/L stock solution of [13C, 15N]TG, 0.5-mL aliquots were acidified with 4 drops of 6 mol/L HCl before extraction three times into 2 mL of ethylacetate. The pooled organic fractions were evaporated to dryness under a steady stream of nitrogen gas at 37°C. Trimethylsilyl (TMS) derivatives were formed by adding 100 μL of bis(trimethylsilyl)trifluoroacetamide containing 10 mL/L trimethylchlorosilane (Supelco, Bellefonte, PA) and heating to 80°C for 30 min. We injected 2-μL aliquots onto a 25 m × 0.2 mm (i.d.) HP5 (cross-linked 5% phenylmethyl silicone) capillary column, fitted into a HP 5970A Mass Selective Detector (both from Hewlett-Packard, Palo Alto, CA). The injector temperature was 260°C, the detector temperature 285°C, and the oven temperature 80°C, programmed to increase at 4°C/min to 275°C, where it remained for 15 min. Injection was in the split mode (~10:1), and detection was in the electron impact mode, scanning the ions at m/z 50 to 500 amu.

Both TG and [13C, 15N]TG produced a single peak under these conditions, corresponding to the mono-TMS derivatives only (Fig. 3). The spectrum for TG is identical to that previously published by Goodman and Markey (8). We confirmed the identity of [13C, 15N]TG by the observation of an identical spectral pattern but with appropriate [M + 2] ions of m/z 156, 172, 216, 231.

Stability and Calibration Curve

The stock aqueous solution of [13C, 15N]TG, 270 μmol/L, was aliquoted into 1-mL volumes and stored at -20°C. No deterioration was noted within 18 months of storage as judged by repeated analysis against freshly made unlabeled TG.

Aqueous preparations of TG were added to urine to provide a series of calibrators with concentrations of 0.1 to 1000 μmol/L. To 0.5 mL of each calibrator we added 10 μL of the stock [13C, 15N]TG (2.7 nmol per tube). TMS derivatives were prepared as described above and analyzed by GC-MS. Data acquisition was in the selected ion monitoring mode: m/z 170, 214, and 229 for unlabeled TG and 172, 216, and 231 for labeled TG. The ratio of ions m/z 170 and 172 was used for quantification.

Analysis for TG in Urine Samples

Urine samples were obtained from a series of 44 controls. This group included urines from normal infants and children (ages 3 months to 16 years, n = 21), normal adults (n = 6), normal newborns (n = 5), and newborns with evidence of asphyxia acidemia (n = 2); postmortem urine demonstrating lactic acidemia (n = 2); urines from fasting ketotic infants (ages 3 months to 1 year, n = 6); and urines from two patients with medium-chain acyl-CoA dehydrogenase deficiency (both homozygous for the A985G mutation). We also obtained urines (disease group) from a patient with classical propionic acidemia (four urine samples) and from a patient with atypical

Fig. 1. Catabolic pathway for isoleucine, showing site of formation of thiglyglycine.

Deficiencies of enzymes named are related to increased thiglyglycine excretion. 2MNH, 2-methyl-3-hydroxybutyryl-CoA; mutase, methylenaloyloxy-CoA mutase.
Fig. 3. (A) Electron impact mass spectrum of TG; (B) regenerated total ion chromatogram of a mixture of TG and $[^{13C}_{3},^{15N}]$TG in approximately equimolar amounts demonstrating purity of products; (C) electron impact mass spectrum of $[^{13C}_{3},^{15N}]$TG.

2-MAA thiolase deficiency (six samples); this second patient had been described previously (L.V. in refs. 9–11). For patients with respiratory chain defects we studied urine from two patients with Pearson syndrome, a disorder presenting with aplastic anemia, neutropenia, and thrombocytopenia associated with deletions of the mitochondrial genome and respiratory chain abnormalities (12); a neonate with complex I deficiency (confirmed by Brian Robinson, Toronto); a 1-year-old infant with a defect between complex I and III (confirmed by John M. Schoffner, Emory University, Atlanta, GA); a patient with partial complex I and complex IV defects; and a patient with cytochrome oxidase deficiency in muscle (the last two confirmed by S. Di Donato, Milan, Italy). Between one and three samples were analyzed for each patient. We performed identical analysis of a single adult urine on five separate occasions to test the reproducibility of the assay. All samples were stored at $-20^\circ$C for as long as 1 year before analysis.

Urine volumes containing 0.1 mg of creatinine (0.89 $\mu$mol) were extracted with ethylacetate containing 2.7 nmol of $[^{13C}_{3},^{15N}]$TG as described for the calibration curve. The selected ion mode was used, as described above.

**Results**

**Linearity and detection limits.** The calibration curve, based on both urine and aqueous solutions, was linear to about 100 $\mu$mol/L (Fig. 4). At greater concentrations there was a lack of linearity, which we attribute to increasing amounts of the natural abundance of the $m/z$ 172 ion. We recommend that appropriate dilutions be made in unknown urines with TG values $>$100 $\mu$mol/L.

The signal-to-noise ratio at 0.1 $\mu$mol/L was $\sim$10:1. Thus, we consider the detection limit for a 0.5-mL sample to be 0.1 $\mu$mol/L.

**Urinary TG concentrations in controls and disease patients.** Fig. 5 shows the values obtained for 44 control subjects: mean value $1.36 \pm 1.12$ mmol/mol creatinine; range 0.14–3.8. One sample analyzed on five separate occasions gave a mean result of 0.74 (SD 0.08) mmol/mol, for a CV of 11%. As shown in Fig. 5 and Table 1, TG concentrations were consistently greater than the controls' in the patients with Pearson syndrome or propi-
otic acidemia and in the patient with 2-MAA thiase deficiency. The concentrations in the four other patients with respiratory chain defects were also increased, but not consistently so in one.

Fig. 6 shows the selected ion chromatogram for m/z 170 and 172 (for TG and [13C,15N]TG, respectively) in a control and an abnormal urine. Positive identification of the correct peak for TG was made upon the correct retention time for a standard TG preparation and from the correct ratio of the m/z 170 ion to the additional ions m/z 214 and 229, as demonstrated in Fig. 3. None of the other peaks with an m/z 170 ion had the characteristic 214 or 229 ions in the correct ratio.

Discussion

We present a method for the accurate quantification of TG in urine by stable isotope dilution GC-MS of TMS derivatives with fragmentation in the electron impact mode. Halvorsen et al. (13) utilized methyl esters for the identification of TG, but their method did not produce a particularly abundant ion suitable for purposes of quantification. The TMS derivatives used here produce high-abundance ions of m/z 170 and 172 for sensitive quantification. We also scanned the ions at m/z 214, 216, 229, and 231, monitoring the relative abundances of each to aid positive identification of TG in the unknown samples. Fig. 6 demonstrates the presence of potentially interfering peaks if we utilized only m/z 170 and 172 for identification of peaks. The incorporation of additional ions is therefore quite important.

The control urinary range of TG varied from 0.14 to 3.84 mmol/mol creatinine, which is within the estimated concentration range of 2.0 mmol/mol provided by Sweetman (14) but lower than the range recently suggested by Guneral and Bachman (15). Neither previous estimate was based on the use of stable isotopes, and both are potentially inaccurate. Our inclusion of ketotic samples and samples from patients with secondary lactic acidemia in the “normal” range study was to control for the ketosis in disorders of isoleucine metabolism and the primary lactic acidemia found in respiratory chain defects. A measurable peak of TG was detectable in all samples analyzed, confirming that the sensitivity of the assay was adequate for use in such studies. Increased TG excretion was detected in a patient with PCC deficiency in samples collected when clinically well and also during metabolic decompensation, when the amount excreted was even higher. The modest increase seen in the patient with 2-MAA thiase deficiency is in keeping with previous experience in this atypical patient, who has high residual enzyme activity and appears to fall into a unique complementation group (10). Previous estimates of TG excretion in complete 2-MAA-thiase deficiency range up to 1000 μmol/L (14). Particularly interesting were the patients with Pearson syndrome, who had consistently high excretion of TG even though neither patient was metabolically stressed. Urinary TG measurement, therefore, should aid the diagnosis of this condition.

The mechanism of the TG increase in all patients with abnormalities of the respiratory chain is probably through secondary inhibition of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase, an NAD+ -requiring dehydrogenase (Fig. 1). NAD+ will become rate-limiting with impaired function of the respiratory chain. Numerous NAD+ -requiring enzyme abnormalities have been im-

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Table 1. Urine tiglyglycine in patients studied.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Diagnosis</th>
<th>TG, mmol/mol creatinine*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Propionic acidemia</td>
<td>103, 497, 183</td>
</tr>
<tr>
<td></td>
<td>(13.3)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2-MAA thiase deficiency</td>
<td>20.7, 30.8, 14.0</td>
</tr>
<tr>
<td></td>
<td>(5.5, 5.9, 5.5)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Pearson syndrome</td>
<td>113, 19.2, 18.6</td>
</tr>
<tr>
<td>4</td>
<td>Pearson syndrome</td>
<td>17.4</td>
</tr>
<tr>
<td>5</td>
<td>Complex I</td>
<td>17.3</td>
</tr>
<tr>
<td>6</td>
<td>Complex I-III</td>
<td>33.8, 0.3, 1.4</td>
</tr>
<tr>
<td>7</td>
<td>Complex I and IV</td>
<td>17.2, 19.9, 4.9</td>
</tr>
<tr>
<td>8</td>
<td>Complex IV</td>
<td>9.7, 5.7</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>0.14–3.8</td>
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* Results for samples collected during patients' illness; results for samples collected when patients were well are indicated in parentheses.
licated secondary to NAD⁺ deficiency due to complex I defects (4, 16). Many, but not all, of the samples from patients with other respiratory chain abnormalities demonstrated above-normal TG concentrations in this study. One patient showed increased concentrations only intermittently. Therefore, although urinary TG measurement is a useful marker in respiratory chain defects, it may require analysis of serial samples.

We conclude that there is great potential for use of this method to discriminate between patients with primary respiratory chain abnormalities and those with secondary lactic acidemia, a discrimination that is at present difficult to achieve.

References