Measurement of Acylcarnitine Substrate to Product Ratios Specific to Biotin-Dependent Carboxylases Offers a Combination of Indicators of Biotin Status in Humans

Anna Bogusiewicz, Thomas D. Horvath, Shawna L. Stratton, Donald M. Mock, and Gunnar Boysen

Abstract
This work describes a novel liquid chromatography tandem MS (LC-MS/MS) method for the determination of ratios of acylcarnitines arising from acyl-CoA substrates and products that reflect metabolic disturbances caused by marginal biotin deficiency. The urinary ratios reflecting reduced activities of biotin-dependent enzymes include the following: 1) the ratio of 3-hydroxyisovalerylcarboxyl-CoA to 3-methylglutaconyl-CoA (3HIAc:MGc) for methylcrotonyl-CoA carboxylase; 2) the ratio of propionylcarboxyl-CoA to propionyl-CoA carboxylase (Pc:Mc) for propionyl-CoA carboxylase; and 3) the ratio of acetylcarboxyl-CoA to 3-methylcrotonyl-CoA carboxylase. To demonstrate the suitability of the LC-MS/MS method for biomonitoring, we measured the ratios for 7 healthy adults at various time points (d 0, 14, and 28) during the induction of marginal biotin through the consumption of egg white. The mean change in the Pc:Mc ratio relative to d 0 was 5.3-fold by d 14 (P = 0.0049) and 8.5-fold by d 28 (P = 0.0042). The mean change in the 3HIAc:MGc ratio was 2.8-fold by d 14 (P = 0.0022) and 3.8-fold by d 28 (P = 0.0001). The mean change in the Ac:Mc ratio was 2.9-fold by d 14 (P = 0.03) and 4.7-fold by d 28 (P = 0.02). The results suggest that simultaneous assessment of ratios of multiple biotin-dependent pathways offers insight into the complex metabolic disturbances caused by marginal biotin deficiency. We hypothesize that one or a combination of the ratios might be more sensitive or robust with respect to other nutrient deficiencies or confounding metabolic processes.

Introduction
Optimal micronutrient nutrition of large populations has been drawing increased attention. However, the limitations of nutrition assessment based on questionnaires and measurement of indices of single micronutrients are well recognized. These include inaccuracy and failure to account for individual variation of micronutrient bioavailability, metabolic requirement, and their interactions.

Biotin is an essential cofactor for 5 known mammalian carboxylases; marginal biotin deficiency causes reduced activity of several of these biotin-dependent carboxylases, which ultimately alters the biochemical flux through these pathways. For example, the reduced activity of one of these, methylcrotonyl-CoA carboxylase (MCC), causes increased urinary excretion of the organic acid 3-hydroxyisovaleric acid (3HIA), a validated indicator of biotin status in humans.

Traditionally, assessment of substrate/product ratios across enzymes that become rate limiting in dedicated metabolic pathways is an excellent way to determine functional enzyme deficiency. Unfortunately, the acyl-CoA substrates and products of the biotin-dependent enzymes are compartmentalized in the mitochondria and are not found in plasma or urine. Fortunately, blocks in acyl-CoA metabolism result in increased urinary excretion of the acylcarnitines corresponding to the accumulating substrates.

Here, we describe the development, analytical performance characteristics, and initial clinical investigation of a liquid chromatography tandem MS (LC-MS/MS) method for the simultaneous determination of urinary substrate/product ratios across the following 3 biotin-dependent carboxylases: 1) MCC (EC 6.4.1.4) is located in the mitochondria and converts 3-methylcrotonyl-CoA to 3-methylglutaconyl-CoA. Accumulating 3-methylcrotonyl-CoA is shunted to 3-hydroxyisovaleryl-CoA and transesterified to 3-hydroxyisovalerylcarboxylate (3HIAc), which is transported out of the mitochondria by the acylcarnitine translocase and into the plasma and excreted in the urine. Increases and decreases in 3-methylglutaconyl-CoA are reflected in an analogous fashion by urinary 3-methylcrotonyl-CoA carboxylase (MCC).
2) Propionyl-CoA carboxylase (PCC; EC 6.4.1.3) is located in the mitochondria and converts propionyl-CoA to methylmalonyl-CoA. Accumulating propionyl-CoA is transesterified to propionylcarnitine (Pc). Changes in methylmalonyl-CoA are reflected in urinary methylmalonylcarnitine (MMc). 3) Both isoforms of acetyl-CoA carboxylase (ACC; EC 6.4.1.2.) convert acetyl-CoA to malonyl-CoA; the related acylcarnitines are acetylcarnitine (Ac) and malonylcarnitine (Mc).

Participants and Methods

**Chemicals.** Optima LC-MS/MS grade water and formic acid (99% pure, Acros Organics) were purchased from Fisher Scientific. Acetyl-L-carnitine hydrochloride reference material was purchased from Sigma-Aldrich. Mc (99% pure), MMc (85% pure), and MGc (95% pure) reference materials were purchased from ChemischeLaboratorien. 3HIAc (98% pure), [N-methyl-D3]-3-hydroxyisovalerylcarnitine ([2H3]-3HIAc, 98% pure), and propionylcarnitine (98% pure) reference materials were all generous gifts from Cambridge Isotope Laboratories.

**Preparation of analytical standards and urine samples.** Analytical standard stock solutions were prepared in water and immediately frozen at −70°C and stored until use. The human urine pool used to assess matrix effects was prepared by taking 40-mL aliquots from fresh, untimed urine samples collected from 6 healthy adult volunteers (4 female, 2 male). After pooling, the urine was thoroughly mixed, aliquots were subdivided into 15-mL tubes, immediately frozen at −20°C and stored until use. Prior to the sample preparation for analysis, all human urines were thawed, warmed to 60°C for 30 min, cooled to ambient temperature, and centrifuged at 3000 × g for 10 min to sediment urine precipitates as previously described (3). The urine supernatant was directly sampled without disturbing the precipitate pellet.

**Clinical study design.** Analyses were performed on urine samples obtained from participants in a study previously reported (4–6). The Institutional Review Board for the University of Arkansas for Medical Sciences approved this study. Written informed consent was obtained from each participant at enrollment and consent was intermittently assessed throughout the study as part of the informed consent process.

To measure the effect of marginal biotin deficiency on the acylcarnitine substrate product ratios for Pc/MMc, 3HIAc/MMc, and AcMc, marginal asymptomatic biotin deficiency was induced in 7 healthy adults (3 women) by feeding a diet low in biotin and high in unenated und egg white for 28 d as previously described (4,5). Leucine intake was controlled by the total protein intake, which was 2.0 g/(kg C176/C176) and stored until use. The human urine pool used to assess matrix effects were also assessed at dilution factors of 5, 10, 25, and 50 for the endogenous levels of Ac, Pc, Mc, MMc, 3HIAc, and MGc present in the urine pool.

**Assessment of urine matrix effect.** Matrix effects were assessed at the 50-fold dilution factor by determining the analytical recovery of [2H3]-3HIAc at concentrations of 12.5, 25, 50, 75, 100, 150, and 200 nmol/L in urine samples and contemporaneously prepared aqueous standards. These measurements were performed on 3 nonsequential days. Urine matrix effects were also assessed at dilution factors of 5, 10, 25, and 50 for the endogenous levels of Ac, Pc, Mc, MMc, 3HIAc, and MGc present in the urine pool.

**Assessment of linearity.** The linearity of aqueous standard curves consisting of 6 points for each of the acylcarnitines was measured to assess the dynamic range for this assay. The dynamic range for each curve was prepared as follows: Ac and MMc, 30–1000 nmol/L; Pc and injection and was held constant for 2 min. Then the percentage of acetonitrile was linearly increased to 95% for the next 4 min and decreased back to 0% after an additional 0.1 min. The column was reequilibrated over the next 3.9 min to complete the 10-min LC cycle. The flow rate was held constant at 200 μL/min throughout the injection cycle.

Retention times for acylcarnitines ranged between 1.26 and 4.06 min for all analyzed samples (Fig. 1). Ac, Pc, Mc, MMc, 3HIAc, and MGc were acquired in selected reaction monitoring mode monitoring the ion transitions of m/z 204 to 85, m/z 218 to 85, m/z 248 to 85, m/z 262 to 85, m/z 262 to 85, and m/z 290 to 85, respectively. The capillary voltage was 4000 V. The desolvation gas temperature and flow rate were 200°C and 11 L/min, respectively. Sheath gas temperature and flow rate were 100°C and 3 mL/min, respectively. Collisionally induced dissociation for all analytes was performed at a collision energy of 20 eV. The retention times for the acylcarnitines were monitored by injection of a mixture of the acylcarnitines over the course of quantitation of all samples; retention times did not exhibit a trend and were reproducible with a SD of <1%.

**Quantitation by LC-MS/MS.** Urine samples were prepared at a 50-fold dilution by mixing 20 μL of sample and 980 μL of water and then vortexed for 10 s. Samples were placed in the autosampler and cooled to 5°C for the duration of analysis. A volume of 2 μL was injected onto a LC-MS/MS system composed of an Agilent 1290 Infinity LC system and an Agilent 6490 triple quadruple mass spectrometer. Chromatographic separations were performed on an Agilent ZORBAX Eclipse Plus-C18, RRHD 2.1 × 100-mm, 1.8-μm analytical column.

The mobile phases were 0.01% formic acid and acetonitrile. The initial mobile phase composition was 0% acetonitrile at the time of
for significance by 1-way ANOVA with repeated measures; if found
MMc, and Ac

Statistical analyses. Differences in the mean urinary 3HIAc:MGc, Pc:
MMc, and Ac:Mc ratios during progressive biotin deficiency were
tested for significance by 1-way ANOVA with repeated measures; if found
significant, Dunnett’s post hoc test was used to identify significant
differences from d 0 using KaliedaGraph (version 4.1.1; Synergy
Software) as previously described (7).

Results and Discussion
Initially, we determined that assessment of any urine matrix
effects on the detection of the 6 acylcarnitines of interest would
be problematic because of the presence of unknown quantities of
the same endogenous acylcarnitines in urine. We avoided this
problem by utilizing [3H3]-3HIAc in diluted urine and water to
determine potential matrix effects on the detection of acylcar-
nitines at the dilution factor of interest. Specifically, the urine
pool described in the “Preparation of analytical standards and
urine samples” section was assumed to contain a broad range of
representative urine constituents found in a diverse population
of participants. Samples were prepared by diluting an aliquot of
the pooled urine 50-fold and adding [3H3]-3HIAc at concen-
trations described in “Participants and Methods.” Signal responses
of the various concentrations of [3H3]-3HIAc from 12.5 to 200
nmol/L in the diluted urine pool relative to aqueous samples
ranged between 100 and 103%; the mean of the CV (%CV) was
3% and all CV were <6%. These observations demonstrate that
urine matrix effects on the determination of [3H3]-3HIAc are
negligible at the 50-fold dilution used in this method and suggest
that the matrix effects for all 6 analyzed acylcarnitines will also
be negligible at this dilution factor.

Urine matrix effects and ion suppression effects were further
evaluated for the 6 endogenous acylcarnitines of interest present
in the urine pool by analysis of the peak areas at 5-, 10-, 25-, and
50-fold dilution. Signal responses were normalized to 100% of
the value at the 50-fold dilution and corrected for the dilution
(Table 1). At the 25-fold dilution, the observed matrix effect was
negligible; in contrast, at the smaller dilution, moderate matrix
effects were observed. We conclude that ion suppression and
other matrix effects of human urine are minimal at 25-fold or
negligible; in contrast, at the smaller dilution, moderate matrix
effects were observed. We conclude that ion suppression and
other matrix effects of human urine are minimal at 25-fold or
greater dilution. This dilution is feasible, because the Agilent

mg per 50 ml

TABLE 1 Endogenous acylcarnitine recoveries from pooled
human urine at various dilutions

<table>
<thead>
<tr>
<th>Urine dilution factor</th>
<th>MGc</th>
<th>3HIAc</th>
<th>MMc</th>
<th>Mc</th>
<th>Pc</th>
<th>Ac</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>25</td>
<td>101</td>
<td>97</td>
<td>95</td>
<td>100</td>
<td>95</td>
<td>103</td>
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<tr>
<td>10</td>
<td>76</td>
<td>71</td>
<td>67</td>
<td>61</td>
<td>71</td>
<td>61</td>
</tr>
<tr>
<td>5</td>
<td>75</td>
<td>71</td>
<td>69</td>
<td>38</td>
<td>91</td>
<td>59</td>
</tr>
</tbody>
</table>

1 Ac, acetylcarnitine; 3HIAc, 3-hydroxyisovalerylcar

TABLE 2 Detectability of acylcarnitines ratios in pooled human
urine at various dilutions

<table>
<thead>
<tr>
<th>Urine dilution factor</th>
<th>3HIAc:MGc</th>
<th>Pc:MMc</th>
<th>Ac:Mc</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>0.78</td>
<td>0.33</td>
<td>23.43</td>
</tr>
<tr>
<td>25</td>
<td>0.75</td>
<td>0.35</td>
<td>22.98</td>
</tr>
<tr>
<td>10</td>
<td>0.73</td>
<td>0.38</td>
<td>23.23</td>
</tr>
<tr>
<td>5</td>
<td>0.74</td>
<td>0.44</td>
<td>36.34</td>
</tr>
</tbody>
</table>

1 Ac, acetylcarnitine; 3HIAc, 3-hydroxyisovalerylcar

TABLE 3 Linear regression parameters obtained from the
calibration curves of acylcarnitines in water

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Slope</th>
<th>Intercept</th>
<th>r2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac</td>
<td>308</td>
<td>913</td>
<td>0.99</td>
</tr>
<tr>
<td>Pc</td>
<td>493</td>
<td>60</td>
<td>0.99</td>
</tr>
<tr>
<td>Mc</td>
<td>126</td>
<td>−81</td>
<td>0.99</td>
</tr>
<tr>
<td>MMc</td>
<td>141</td>
<td>−342</td>
<td>0.99</td>
</tr>
<tr>
<td>3HIAc</td>
<td>846</td>
<td>949</td>
<td>0.99</td>
</tr>
<tr>
<td>MGc</td>
<td>284</td>
<td>158</td>
<td>1.00</td>
</tr>
</tbody>
</table>

1 Ac, acetylcarnitine; 3HIAc, 3-hydroxyisovalerylcar

Acylcarnitine ratios indicative of biotin status 1623

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ical interest, because a similar degree of deficiency induced in pregnant mice causes cleft palate and limb shortening defects in 100% of the pups (10, 11).

The 3 ratios tended to increase with time consuming the egg white diet relative to d 0 (Fig. 2A–C). When calculated for each participant relative to that person’s value on d 0, the mean change in the Pc:MMc ratio was 5.3-fold by d 14 (P = 0.0049) and 8.5-fold by d 28 (P = 0.0042). The mean change in the 3HIAc:MGc ratio was 2.8-fold by d 14 (P = 0.0022) and 3.8-fold by d 28 (P = 0.0001). The mean change in the individual Ac:Mc ratio was 2.9-fold by d 14 (P = 0.03) and 4.7-fold by d 28 (P = 0.02). For comparison, data are provided for the PCC activity measured in lymphocytes isolated from peripheral blood for each participant (Fig. 2D) and the diagnostic sensitivity at d 14 and d 28 (percent abnormal) is provided for these 4 indices plus 2 others (urinary 3HIA and 3HIAc) in Table 5. For interest, we included the data from a participant who admitted noncompliance on questioning at the end of the study (6). That participant’s PCC activity did not decrease to less than the lower limit of normal and that participant’s 3 ratios all remained within the normal range at d 14 but increased to greater than the upper limit of normal by d 28. One interpretation is that PCC activity will prove to be the most robust for detecting marginal biotin deficiency; an alternative interpretation is that the noncompliant participant did become biotin deficient, but to a lesser degree, and that the abnormal ratios reflect this subtle metabolic block. Because the biotin-dependent enzymes whose substrate:product ratios were examined here (MCC, PCC, and ACC-1 or ACC-2) are at the heart of intermediary metabolism, the fluxes down any one of these pathways are determined by multiple other processes (e.g., dietary intake of several macro-

### Table 4

<table>
<thead>
<tr>
<th>Analyte:analyte ratio</th>
<th>Intra-day</th>
<th>Inter-day</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Ac</td>
<td>0.4</td>
<td>4.7</td>
</tr>
<tr>
<td>Pc</td>
<td>2.8</td>
<td>4.5</td>
</tr>
<tr>
<td>Mc</td>
<td>2.2</td>
<td>8.5</td>
</tr>
<tr>
<td>MMc</td>
<td>1.7</td>
<td>3.7</td>
</tr>
<tr>
<td>3HIAc</td>
<td>6.7</td>
<td>7</td>
</tr>
<tr>
<td>MGc</td>
<td>2</td>
<td>4.5</td>
</tr>
<tr>
<td>Ac:Mc</td>
<td>2.2</td>
<td>4.2</td>
</tr>
<tr>
<td>Pc:MMc</td>
<td>3.8</td>
<td>3.8</td>
</tr>
<tr>
<td>3HIAc:MGc</td>
<td>8.4</td>
<td>7.9</td>
</tr>
</tbody>
</table>

1 Ac, acetylcarnitine; 3HIAc, 3-hydroxyisovaleryl carnitine; Mc, malonylcarnitine; MGc, 3-methylglutaryl carnitine; MMc, methylmalonylcarnitine; Pc, propionylcarnitine.

2 n = 8.

3 n = 24.

### Table 5

<table>
<thead>
<tr>
<th>Indicator of biotin status</th>
<th>Abnormal on d 14</th>
<th>Abnormal on d 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urinary 3HIAc:MGc</td>
<td>86</td>
<td>86</td>
</tr>
<tr>
<td>Urinary Ac:Mc</td>
<td>71</td>
<td>71</td>
</tr>
<tr>
<td>Urinary Pc:MMc</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Lymphocyte PCC activity</td>
<td>86</td>
<td>86</td>
</tr>
<tr>
<td>Urinary 3HIA carnitine excretion</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Urinary 3HIA excretion</td>
<td>86</td>
<td>86</td>
</tr>
</tbody>
</table>

1 Defined as the percentage of participants whose values were abnormal (consistent with marginal biotin deficiency) by d 14 and 28. Ac, acetylcarnitine; 3HIA, 3-hydroxyisovaleric acid; 3HIAc, 3-hydroxyisovaleryl carnitine; Mc, malonylcarnitine; MGc, 3-methylglutaryl carnitine; MMc, methylmalonylcarnitine; Pc, propionylcarnitine; PCC, propionyl-CoA carboxylase.

![Figure 2](https://academic.oup.com/jn/article-abstract/142/9/1621/4630888)
method development and provided the required equipment for analyses. All authors read and approved the final manuscript.

**Literature Cited**


2. Roe CM, Millington DS, Matby DA. Identification of 3-methylglutarylcar


