Measurement of 3-Hydroxyisovaleric Acid in Urine of Biotin-Deficient Infants and Mice by HPLC

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ABSTRACT We developed an assay for measuring urinary 3-hydroxyisovaleric acid (3-HIA) using HPLC after derivatization with 2-nitrophenylhydrazine hydrochloride (2-NPH·HCl). The derivatized 3-HIA was extracted into n-hexane and separated isocratically on a C8 reversed-phase column for fatty acids (YMC-Pack FA). We used this method to measure 3-HIA in urine extracts from mice fed a biotin-deficient diet for >4 wk and in an infant who was fed a special Japanese formula and was suspected of being biotin deficient. Urinary 3-HIA could be assayed within the range of 0.42–8.5 mmol/L with high accuracy by this method, as an indicator of biotin deficiency. Therefore, the HPLC method for 3-HIA described here may be a useful tool clinically as well as in the research laboratory. J. Nutr. 135: 615–618, 2005.

KEY WORDS: • 3-hydroxyisovaleric acid • HPLC • biotin deficiency • methylcrotonyl CoA carboxylase • urine

Biotin is a cofactor for several carboxylases used in fatty acid synthesis, gluconeogenesis, and BCAA metabolism. The major biotin-containing enzymes are β-methylcrotonyl-CoA carboxylase (MCC),3 propionyl-CoA carboxylase, pyruvate carboxylase, and acetyl CoA carboxylase. MCC catalyzes an essential step in the degradation of leucine, which converts β-methylcrotonyl-CoA to 3-methylglutaconyl-CoA. The reduced activity of MCC leads to an elevated excretion of 3-methylcrotonic acid, the product of its hydration (3-hydroxyisovaleric acid: 3-HIA), and 3-methylcrotonylglycine, formed by conjugation with glycine. The increased urinary excretion of these normal metabolites reflects reduced activity of MCC or is due to dietary biotin depletion in genetically normal individuals.

Conditions that cause biotin deficiency include the following: long-term consumption of undercooked egg white; infant diets that do not include biotin; deficiency of one or more of the biotin-dependent carboxylases in inherited metabolic disorders; biotinidase deficiency and holocarboxylase synthetase deficiency; and biotin transporter deficiency (1,2). One of the most important criteria for the diagnosis of biotin deficiency is the detection of organic acids in urine, such as 3-HIA and 3-hydroxypropionic acid, which are elevated in biotin deficiency (3–5).

In general, 3-HIA in the urine and/or serum of biotin-deficient patients and experimental animals has been assayed by GC/MS, which is specific for assaying 3-HIA in screening tests for biotin deficiency. However, the procedure is complex and difficult. Therefore, we devised an assay for urinary 3-HIA by HPLC, using equipment that is available in most laboratories. We also discuss the usefulness of this method for assaying 3-HIA in the urine of biotin-deficient and biotin-supplemented mice and human infants.

MATERIALS AND METHODS

Animal care and urine collection. Male mice (ICR/Jcl) were obtained from CLEA Japan at 8 wk of age. The biotin-deficient diet was purchased from Oriental Yeast in pelleted form. The components of the biotin-deficient diet (g/kg) were as follows: egg white, 245; cornstarch, 465; sucrose, 100; nonnutritive cellulose, 50; corn oil, 60; mineral mix, 70; and vitamin mix, 10. The control diet was made by supplementing the biotin-deficient diet with biotin (5.0 mg of biotin/kg diet). Mice were kept in an animal room maintained at constant temperature (23 ± 2°C) with a 12-h light:dark cycle (0700–1900 h).

The mice consumed a biotin-deficient or biotin-supplemented diet ad libitum and had free access to distilled water for 6 wk. The body weights of the 14-wk-old mice were 27.9 ± 2.5 and 28.2 ± 2.0 g in the biotin-deficient and biotin-supplemented groups, respectively. Consumption of the diet was also confirmed to be approximately the same in both diet groups, as shown in our previous study (6). Urine was collected from 3 mice fed the biotin-deficient or biotin-supplemented diet every week for 6 wk. The urine of the mice in the individual metabolic cages was collected for 24 h and stored at −40°C until use. All procedures were performed in accordance with the standards related to the care and management of experimental animals of the Japanese Prime Minister’s Office (7).

Urine collection in an infant. Because biotin is not yet registered in Japan as a food additive, except in some foods such as dietary supplements, it cannot be added to infant formulas. We demonstrated previously that the biotin concentration of special formulas for medical treatment and prevention of disease in Japan was less than a fifth of the level in American products (8). Therefore, it has often been
reported that biotin deficiency develops in infants with food allergies or inborn errors of metabolism who have been fed special Japanese formulas (9).

In the present case, the infant studied was a 5-month-old Japanese male. At 4 wkts of age, he was diagnosed as having dyspepsia and started to receive maternal milk and/or special formula called Elemental Formula (Meiji Milk Products). Afterwards, prominent erythematous skin lesions developed on his eyelids, perioral region, and neck. Dietary biotin deficiency was strongly suspected and oral treatment with 1 mg biotin was started. After 2 wkts, the skin lesions disappeared rapidly, and the infant recovered and remained well. Urine was collected 1 wk before and after the biotin treatment. This study was performed in accordance with the ethical principles for medical research involving human subjects (Declaration of Helsinki, World Medical Association). Informed consent was obtained from the parents at enrollment.

Reagents. For the HPLC analysis of standards, 3-HIA was obtained from Tokyo Kasei Kogyo. A kit (X8RFAR 01) for the analysis of short- and long-chain FFA by HPLC (YMC) was used for the pretreatment of the 3-HIA standard solution and urine samples. This kit contains a derivatized reagent that converts the carboxyl moiety of FFA into 2-nitrophenylhydrazide (2-NPH). Sensitive detection in the UV-visible range thus becomes possible after a simple derivatization procedure. Analytical reagent-grade acetonitrile was obtained from Wako Chemical Industries. The concentrations of urinary biotin and 3-HIA were measured in all urine samples with the picric acid method using a Creatinine Test Wako kit (Wako Chemical Industries). The microbioassay lacks specificity compared with biotin analysis using an HPLC/streptavidin assay. Urinary specimens were filtered through 0.45-μm membranes and assayed without hydrolysis. The creatinine concentrations were measured in all urine samples with the picric acid method using a Creatinine Test Wako kit (Wako Chemical Industries). The concentrations of urinary biotin and 3-HIA were expressed as μmol/mol creatinine and mmol/mol creatinine, respectively.

Statistical analyses. For statistical evaluation of the data in mice, repeated-measures ANOVA and Student’s t test were used. Differences of P < 0.05 were considered significant. StatView 5.01 (SAS Institute) software was used for all statistical analyses. Values in the text are means ± SD.

RESULTS

In the chromatograms of the 3-HIA standard solution and of the urine analyzed by HPLC, the peak of authentic 3-HIA was identified as the position with an RT of 8.32 min (Fig. 1a). In the chromatogram of the extract of the urine of mice fed a biotin-deficient diet for 6 wks, a high peak was detected at 8.33 min and low unknown peaks appeared (Fig. 1b). 3-HIA was
separated completely from other fatty acids in each sample. A peak was detected at 8.72 min in the urine of the biotin-supplemented mice at a position markedly different from the peak of 3-HIA (Fig. 1c). No peak was present at 8.33 min.

Standard 3-HIA concentrations ranging from 0.42 to 8.5 mmol/L and the peak area were correlated \( (r = 0.999, P < 0.01) \) (Fig. 2a). In mice fed the biotin-deficient diet for 6 wk, the concentration of urinary 3-HIA was 114.2 ± 69.6 mmol/mol creatinine, which was higher than the concentration of 35.2 ± 23.8 mmol/mol creatinine in mice fed the biotin-deficient diet for 4 wk \( (P < 0.046; \text{Table 1}) \). However, no profound clinical signs of biotin deficiency, such as loss of hair or dermatitis, were observed in these biotin-deficient mice during the experimental period.

3-HIA was detected by HPLC in the urine of biotin-deficient mice (3-HIA-positive mice) (Table 1). The incidence of 3-HIA-positive mice increased with the length of time the biotin-deficient diet was fed. There were no 3-HIA-positive mice within 3 wk of feeding, but all biotin-deficient mice excreted 3-HIA in their urine after 4 wk of feeding. Urinary biotin and 3-HIA concentrations in biotin-deficient mice were inversely correlated \( (r = -0.58, P = 0.021; \text{Fig. 2b}) \). When the biotin concentration in the urine was <15 μmol/mol creatinine, 3-HIA was detected in the urine of 6 of 7 mice. On the other hand, no 3-HIA was detected in the urine of 8 biotin-deficient mice that had a urinary biotin level > 15 μmol/mol creatinine. Moreover, no 3-HIA was detected in the urine of the biotin-supplemented mice (Table 1).

The chromatogram of urine obtained from the infant fed a special formula manufactured in Japan and suspected of being biotin deficient had its highest peak at 8.27 min (Fig. 1d). Another peak was detected at 8.77 min. However, these peaks were not detected in the urine after biotin treatment (Fig. 1e).

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**TABLE 1**

Effects of biotin deficiency on the excretion of 3-HIA in urine of mice

<table>
<thead>
<tr>
<th>Duration of feeding</th>
<th>Mice excreting 3-HIA/mice examined</th>
<th>3-HIA (mmol/mol creatinine)</th>
<th>Biotin (μmol/mol creatinine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n/n</td>
<td></td>
<td>6.9</td>
<td>114.2 ± 69.6</td>
</tr>
<tr>
<td>1</td>
<td>0/3</td>
<td>2.2</td>
<td>22.3 ± 3.6</td>
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<td>2</td>
<td>0/3</td>
<td>2.2</td>
<td>29.7 ± 10.3</td>
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<tr>
<td>3</td>
<td>0/3</td>
<td>16.5</td>
<td>16.5 ± 12.6</td>
</tr>
<tr>
<td>4</td>
<td>2/3</td>
<td>35.2 ± 23.8</td>
<td>5.9 ± 5.3</td>
</tr>
<tr>
<td>6</td>
<td>3/3</td>
<td>114.2 ± 69.6</td>
<td>2.8 ± 2.2</td>
</tr>
<tr>
<td>Biotin supplemented</td>
<td></td>
<td>23.8 5.9</td>
<td>263 ± 175</td>
</tr>
<tr>
<td>1</td>
<td>0/3</td>
<td>ND</td>
<td>772 ± 476</td>
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<tr>
<td>2</td>
<td>0/3</td>
<td>ND</td>
<td>1020 ± 680</td>
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<td>3</td>
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<td>ND</td>
<td>591 ± 387</td>
</tr>
<tr>
<td>6</td>
<td>0/3</td>
<td>ND</td>
<td>493 ± 319</td>
</tr>
</tbody>
</table>

1 Values are n or means ± SD.

2 ND, not detected.

3 3-HIA was positive in only 1 mouse with a urinary level of 12.0 mmol/mol creatinine, a value below the limit of detection.

**DISCUSSION**

GLC was used previously to detect 3-HIA and β-methylcrotonylglycine after hydrogenation of urinary extracts from humans with MCC deficiency (12). Subsequently, the first application of GC/MS for the quantitative detection of 3-HIA in urine was reported by Mock et al. (13). Derivatized 3-HIA was detected in urine extracts of patients with biotin deficiency. However, in addition to the high cost of the necessary equipment, after repeated extraction and the separation of the organic acids in the urine, a derivative for separation by GC must also be made (14). In addition, measurement of urinary 3-HIA is a time-consuming process.

Several HPLC methods were developed for the analysis of fatty acids in serum and urine; these employ preclusion derivatization techniques to increase the sensitivity and specificity of detection (10,11,15,16). In the present study, the pre-processing, including labeling, was simplified by the use of the derivatization technique, and a method for 3-HIA measurement was established using a standard HPLC system. The lower limit of detection of urinary 3-HIA was 0.042 mmol/L. This sensitivity was sufficient to measure 3-HIA in urine samples from an infant suspected of being biotin deficient. A relatively higher peak of 3-HIA was obtained before biotin treatment compared with other fatty acids present in the urine. These findings suggest that this HPLC method is sufficiently sensitive for assaying urinary 3-HIA in screening tests for biotin deficiency in humans.

The recovery of 3-HIA was in the range of 90.1–108.8%. This indicates that the present method does not have completely satisfactory precision for analyzing the 3-HIA concentration in urine because the method used here has several extraction and separation steps, which may affect the recovery of 3-HIA. The normal range of urinary 3-HIA in humans was reported to be from 5.1 to 10.7 mmol/mol creatinine as assessed by GC/MS, and it is increased several-fold by biotin deficiency (17). In the present study, the urinary 3-HIA concentration was 78.6 mmol/mol creatinine in an infant fed a special Japanese formula. However, no 3-HIA was detected after biotin treatment of this infant.
The concentrations of biotin and organic acids, and the activity of carboxylase in the serum and urine are generally used as indicators of biotin status (18,19). Mock et al. (3–5) demonstrated that decreased urinary biotin and increased urinary 3-HIA are sensitive indicators of early biotin deficiency, but methylcrotonylglycine and isovalerylglycine, which are also produced due to the decreased activity of MCC, are not. 3-HIA is detected in urine before the appearance of clinical signs of biotin deficiency; thus, it is expected to be a useful indicator of early biotin deficiency. The measurement of 3-HIA in urine may thus be useful for the diagnosis of biotin deficiency.

3-HIA is also a sensitive indicator of biotin deficiency in mice. 3-HIA was detected within a short time after the beginning of the feeding of a biotin-deficient diet, along with a decrease of biotin in the urine. Thus, the HPLC method for 3-HIA described here can be a useful tool clinically as well as in the research laboratory.

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LITERATURE CITED