Original Article

Serum biotin in Japanese children: Enzyme-linked immunosorbent assay measurement

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Abstract Background: Biotin deficiency has been reported in Japanese infants fed special formulas for medical reasons, including those with milk allergy and congenital metabolic diseases, because these formulas contain little biotin. Serum biotin measurement is useful for diagnosing biotin deficiency. We applied a simple and rapid method to analyze serum biotin, and established normal ranges for children and adults.

Methods: Serum biotin in 188 healthy Japanese children aged 0–4 years and in 25 healthy adults was analyzed using a Biotin ELISA Kit (immundiagnostik). The effects of various conditions on the measurement of serum biotin were also examined.

Results: Median biotin in children aged 0–4 years was 10.4 ng/dL (IQR, 7.9–13.4 ng/dL), and that in adults was 12.9 ng/dL (IQR, 10.8–15.8 ng/dL). Normal range was 4.7–22.0 ng/dL in children and 8.4–20.5 ng/dL in adults (calculated using two-sided 95% CI). Measurements obtained with this method were not affected by frozen storage, freeze–thaw, or hemolysis, indicating that serum biotin can be analyzed accurately under these conditions, with a possible application to plasma samples.

Conclusions: Serum biotin was significantly lower in children than in adults, with the normal range being 4.7–22.0 ng/dL in children and 8.4–20.5 ng/dL in adults. This simple and accurate enzyme-linked immunosorbent assay method is useful for diagnosing biotin deficiency.

Key words adult, child, enzyme-linked immunosorbent assay, infant, serum biotin.

Biotin is an essential, water-soluble vitamin that acts as a cofactor for five carboxylases.1 Dietary reference intakes for Japanese (2015) specified by the Minister of Health, Labour and Welfare set the adequate intake (AI) of biotin at 4 μg/day for infants in the first 6 months of life, based on the average biotin intake of breast-fed infants.2 AI was extrapolated to 10 μg/day for infants aged 6–11 months, 20 μg/day for 1–5-year-old children, and gradually increased to 50 μg/day for adults,2 although a small amount of biotin is synthesized by intestinal bacteria and absorbed.3 Recently in Japan, biotin deficiency has been reported in infants fed special formulas for medical reasons, including those with milk allergy and congenital metabolic disease,4–13 because these formulas contain little biotin.14 Biotin deficiency can lead to reduced methylcrotonyl-CoA carboxylase activity, which causes an increase in urinary 3-hydroxyisovaleric acid (3HIA). Although increased urinary 3HIA secretion and decreased urinary biotin excretion are reportedly more sensitive indicators of biotin deficiency,5,14,15 than serum biotin, measurement of urinary 3HIA and of biotin require great care.15–19 Watanabe et al. reported a bioassay for serum biotin,20 but analysis of this method involves an immense amount of time and effort. In the present study, we used a biotin enzyme-linked immunosorbent assay (ELISA) kit, a simple and rapid method for analysis of serum biotin under various conditions, and established normal ranges for children and adults.

Methods

Samples

Fresh sera were obtained from three healthy adults for studying the effects of various parameters on biotin assay. Frozen stored, freeze-thawed, and partially hemolyzed sera, as well as plasma, were used.

Sera from 188 healthy children (0–4 years old) and 25 healthy adults not taking supplements were analyzed to establish the normal ranges of serum biotin in children and adults.

This study was approved by Teikyo Heisei University Review Board (No. 26–001-1), Teikyo University Review Board (No.12–117-3), and Juntendo University Hospital Review Board (No.25–303). Written informed consent was obtained from each child’s parents and from the adult participants.

Serum biotin

Serum biotin was analyzed using a commercially available biotin ELISA kit (K8140; Immundiagnostik, Bensheim, Germany) according to the manufacturer’s procedure. Each serum sample (150 μL) was added to individual wells. Serum samples and standard biotin solutions were pre-incubated with 150 μL streptavidin-enzyme conjugate solution for 15 min at 25°C. After stopping the reaction by adding 100 μL 0.12 mol/L hydrochloric acid, the plates were incubated at 4°C for 10 min. The plates were then washed for 3 times with PBS (pH 7.6). Streptavidin-HRP conjugate solution was added to the wells and incubated for 30 min at room temperature. After washing for 3 times, 3,3′,5,5′-tetramethylbenzidine substrate solution (100 μL) was added to each well. Incubation was continued for 30 min. The reaction was stopped with 40 μL of 2N硫酸. The absorbance at 450 nm was measured using an automatic plate reader. The biotin concentration was calculated from the standard curve generated using the absorbance.

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acid to each well, absorbance at 450 nm was measured using a plate reader (iMark microplate reader, Bio-Rad, Hercules, California, USA). Absorbance was calculated by subtracting the reference absorbance at 620 nm from that at 450 nm. The range 3.8–30 ng/dL was adopted for measurement of biotin. When biotin was >30.0 ng/dL, the sample was diluted to an adequate concentration with a dilution solution included in the kit.

Recovery rate of biotin added to the serum
Biotin solution (25 μL, 60.0 ng/dL) was added to 125 μL sera from each of three healthy adults. Biotin in these sera was determined as described here, in parallel with sera to which no biotin was added.

Stability under freeze–thawing
A portion of each serum sample was kept at −20°C for 5 months in a freezer. Biotin in these sera was compared with that in samples that had not been frozen. Changes in biotin level in samples treated by repeated freeze–thaw cycles were also examined.

Effect of hemolysis
A portion of withdrawn blood from three healthy adults was sonicated for 10 min. Biotin of sonicated sera was compared with samples without sonication.

Possible binding of biotin to serum protein(s)
A portion of freshly prepared sera obtained from three healthy adults was mixed with an equal volume of 4.5 N sulfuric acid and autoclaved at 121°C for 60 min under 121×10^5 N/m². After cooling, the treated sera were neutralized by addition of 4.5 N sodium hydroxide according to Oyamada et al. Biotin bound with serum protein(s) was estimated before and after acid treatment.

Biotin in plasma and serum
Plasma and serum were prepared from freshly prepared blood with or without EDTA-2 K, respectively. Biotin in both samples was determined as described in the previous section.

Normal range of serum biotin
The sera obtained from 188 children aged 0–4 years were stored in a freezer, and the biotin level determined as described (Fig. 1). Sera obtained from 25 healthy adults aged 21–50 years (19 men, six women) were also stored in a freezer at −20°C. The biotin level of these samples was also determined as described (Fig. 1).

Statistical analysis
Significant differences between each age group of children, and between children and adults were examined using Wilcoxon rank sum test. Two-sided 95% CI were adopted as the normal range for children and adults according to the standardization method proposed by the Japanese Committee For Clinical Laboratory Standards.

Results
Recovery rate of biotin added to serum
In the recovery test, 101.4% of added biotin was recovered (individual data: 95.5%, 104.0% and 104.7%), indicating the absence of factors interfering with the measurement of biotin in serum.

Stability of biotin in frozen stored serum
Mean biotin in serum stored for 5 months in a freezer was 99.9% of the control serum level (individual data: 97.9%, 99.2% and 102.5%), indicating that serum biotin is stable under frozen storage.

Stability of biotin in freeze–thawed serum
Mean biotin in freeze–thawed serum was 102% that of untreated serum (individual data: 97.8%, 102.6% and 106.9%), and the mean biotin level in repeated freeze–thawed serum was 100% (94.8%, 100.6% and 104.6%) of that of untreated serum. This shows that freeze and thaw treatments do not interfere with the measurement of serum biotin.

Effect of hemolysis on serum biotin
Mean biotin level in sonicated serum was 101.4% compared with control samples (individual data: 97.8%, 102.5% and 104.0%), indicating that hemolysis also has no influence on serum biotin analysis.
Biotin in serum and plasma

Mean biotin in plasma was 97.7% of that in the serum (individual data: 93.2, 98.2 and 101.6%), indicating that plasma can also be used for biotin analysis.

Biotin bound to serum proteins

As shown in Table 1, serum biotin increased to almost double due to acid hydrolysis, suggesting that approximately half the biotin in the serum is bound to serum proteins.

Normal range of serum biotin

There was no significant difference in biotin level between children in the age-specific groups (Fig. 1a), therefore, data from all 0–4-year-olds were combined and compared with those of adults (Fig. 1b). Median biotin level in children aged 0–4 years was 10.4 ng/dL (IQR, 7.9–13.4 ng/dL), and 12.9 ng/dL (IQR, 10.8–15.8 ng/dL) in adults. Serum biotin in children was significantly lower than in adults ($P = 0.00188$).

Figure 2 shows the distribution of serum biotin in children aged 0–4 years. Calculating a two-sided 95% CI gave a normal range of biotin in children as 4.7–22.0 ng/dL. The normal range in adults was calculated as 8.4–20.5 ng/dL (Fig. 3).

Serum biotin at 0–6 months vs feeding method

There was no significant difference in serum biotin level in infants fed with breast milk, artificial milk or mixed milk (Fig. 4).

Table 1  Biotin level in acid-treated serum (untreated serum)

| Sample | Serum biotin (ng/dL) | % of control |
|--------|---------------------|-------------|
| A      | Untreated           | 12.2        | –           |
|        | Acid treated        | 24.2        | 198.4       |
| B      | Untreated           | 20.5        | –           |
|        | Acid treated        | 38.0        | 185.4       |
| C      | Untreated           | 15.8        | –           |
|        | Acid treated        | 36.9        | 233.5       |

Discussion

Median serum biotin levels measured using a commercially available ELISA kit was 10.4 ng/dL (IQR, 7.9–13.4 ng/dL) in children 0–4 years of age and 12.9 ng/dL (IQR, 10.8–15.8 ng/dL) in adults, showing that serum biotin in young children is significantly lower than in adults. This may be due to differences in biotin intake or intestinal bacterial flora between children and adults.23

Suzuki et al. noted that mean serum biotin was 14.2 ± 6.3 ng/dL in elementary school children using the same method.24 Mock and Malik used an avidin-binding assay with prior high-performance liquid chromatography separation and reported 391 ± 146 pmol/L (equivalent to 9.5 ± 3.6 ng/dL) for adults.18 The present results are in line with these previous data.

Serum biotin measured on ELISA was not affected by frozen storage, freeze–thaw, or hemolysis, indicating that serum under these conditions can be reliably analyzed. Plasma can also be used for ELISA measurement of biotin. In addition, this method is very simple, accurate, and easy to access in clinical practice.

No significant difference was observed in biotin level between infants fed breast milk and artificial milk. The biotin content in breast milk is reported to be 0.5 μg/dL, which is similar to the content in artificial milk (0.4–1.0 μg/dL).25 The same normal range...
of serum biotin can therefore be applied to infants fed breast milk and those fed artificial milk.

Biotin serum increases significantly when serum is hydrolyzed with hydrochloric acid. This strongly suggests that some biotin in serum is bound to serum proteins. The present ELISA results are consistent with this finding. Biotin in serum increased substantially with acid hydrolysis, showing that biotin is bound to serum proteins and cannot be measured with ELISA without acid hydrolysis.

Serum biotin has been measured previously using bioassay methods. The normal range of free biotin for infants fed breast milk, artificial milk and mixed milk have been reported to be 0.6 ± 0.3 ng/mL, 0.8 ± 0.4 ng/mL and 0.6 ± 0.1 ng/mL, respectively. The normal range of serum biotin has also been reported for children (0.4–1.1 ng/mL) and healthy adults (1.9 ± 1.4 ng/mL and 0.4–1.1 ng/mL). These levels are significantly higher than those obtained on ELISA, including the present results, most likely due to the difference in measurement method.

Biotin deficiency has been reported in infants fed special formulas for medical reasons, including milk allergy and congenital metabolic diseases. We examined serum biotin level on ELISA in two infants who were fed special formulas for milk allergy and who were suspected to have biotin deficiency based on hair loss. Serum biotin in these two patients was 1.1 ng/dL and 0.7 ng/dL, respectively, which is significantly lower than the normal range observed in the present study, indicating that they were suffering from biotin deficiency. These patients received biotin, and the hair loss promptly improved. One of the patients was treated with oral biotin 1 mg/day for 2 weeks, resulting in an increase in serum biotin, from 0.7 ng/dL to 120 ng/dL; this dosage was too high, although no adverse effects of biotin treatment were observed. This suggests that measurement of serum biotin will also be useful for determining the appropriate dosage of biotin once biotin deficiency is treated.

Stratton et al. reported that urinary 3HIA is useful for diagnosing marginal biotin deficiency. Patients presenting with symptoms of biotin deficiency all had elevated urinary 3HIA, whereas serum biotin was normal in some patients. Although urinary 3HIA may serve as a better indicator of biotin deficiency, this simple serum biotin measurement method will offer an alternative and useful diagnostic tool for biotin deficiency.

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Disclosure
The authors declare no conflict of interest.

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