A Case of β-Carotenemia Misdiagnosed as Jaundice by the Bilirubin Oxidase Method

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Summary Serum bilirubin measurement is necessary to accurately distinguish jaundice from carotenemia. A 59.8-y old Japanese male showed symptoms of yellow skin pigmentation as a result of β-carotenemia. Diagnostic laboratory results indicated elevated levels of serum muscle enzymes (aspartate aminotransferase, lactate dehydrogenase, and creatine kinase), but normal levels in liver function tests (alanine aminotransferase and direct bilirubin). The laboratory results indicated hypothyroid myopathy. Moreover, although the patient did not show significant abnormalities in liver function tests, the serum level of total bilirubin (TBIL) measured by bilirubin oxidase method was markedly increased beyond the upper limit of normal. Fundamental experiments revealed that the bilirubin oxidase method had a positive interference by β-carotene. These findings suggested that hyper β-carotenemia could have caused the falsely elevated serum TBIL levels in the patient.

Key Words carotene, bilirubin, hypothyroidism, yellowish skin pigmentation, liver disease, myopathy

Note

Usually, yellowish skin pigmentation indicates presence of either jaundice or carotenemia. The former is diagnosed by elevated levels of serum total bilirubin (TBIL) and marked by yellowness of the sclera (white layer of the eye), while carotenemia is diagnosed by elevated levels of serum carotenoids (e.g., β-carotene or cryptoxanthin) without any yellow coloration of eyes (1). However, we encountered a patient suffering from β-carotenemia (8.16 μmol/L, reference range 0.02–2.14 μmol/L) with an elevated serum TBIL (24.0 μmol/L by bilirubin oxidase method (2), reference range 3–19 μmol/L) and no indication of yellowish sclera. Currently, a common understanding is that serum TBIL levels measured by the traditional diazo method are normal in patients with β-carotenemia. The above finding is confusing to physicians while distinguishing jaundice from β-carotenemia. In this study, we highlight an issue that β-carotenemia in some cases is misdiagnosed as jaundice by the bilirubin oxidase (BOX) method. We further investigated the ingredient in the bilirubin assay reagent that reacts with β-carotene and causes discrepancy.

Patient and Methods

Case presentation. The patient was a 59.8-y old Japanese male. He was admitted for the evaluation of yellowish skin pigmentation. Except controlled diabetes mellitus, there were no notable diseases observed in his medical history, but laboratory examinations at the first visit revealed that serum enzyme levels were high: aspartate aminotransferase (AST, 40 U/L), lactate dehydrogenase (LD, 589 U/L), and creatine kinase (CK, 339 U/L), along with a slight elevation in the serum TBIL (24.0 μmol/L) measured by the BOX method. However, serum direct bilirubin (DBIL, 1.7 μmol/L; reference range, <3.4 μmol/L) and alanine aminotransferase (ALT, 20 U/L) were not elevated. Supplementary examinations of free triiodothyronine (free T3, 1.47 pmol/L), free thyroxine (free T4, 1.78 pmol/L), and thyroid stimulating hormone (TSH, 174 μU/mL) confirmed the presence of hypothyroidism in the patient. Serum β-carotene concentration was found to be elevated (8.16 μmol/L). Based on the above examinations, the patient was diagnosed with β-carotenemia potentially due to hypothyroidism (3). β-Carotenemia can occur without the consumption of carotenoid-rich foods, such as in patients with liver disease, diabetes mellitus, nephrotic syndrome, anorexia nervosa, and patients with hypothyroidism.

Assays. The recruitment of sufficient number of healthy subjects with primary β-carotenemia caused by overconsumption of carotenoid-rich foods was challenging. As a result, serum samples were obtained from 42 patients with overt (n=15) and subclinical (n=27) hypothyroidism that had visited Toho University Ohashi
Medical Center. In accordance with the Helsinki Declaration, informed consent was strictly observed and the study was approved according to the guidelines established by the Protection of Human Subjects Committee of Toho University Ohashi Medical Center (Ethics Committee approval number 14-54: October 14, 2014). A solution of β-carotene was prepared as a surrogate of native β-carotene present in serum according to the method described by Tsushida et al. (4). In brief, 0.25 mL of β-carotene (4 mg in 5 mL chloroform) was added to the mixed solution of 0.1 mL of linoleic acid (0.555 mL in 5 mL chloroform) and 0.5 mL of Tween-40 (1 g in 5 mL chloroform), followed by evaporation with nitrogen gas. The resultant pellet was dissolved in 18 mL of 0.1 mol/L phosphate buffer (pH 6.8) and 2 mL of distilled water.

Table 1. Serum β-carotene and TBIL concentrations in patients with hypothyroidism.

<table>
<thead>
<tr>
<th></th>
<th>β-Carotene μmol/L</th>
<th>BOX-TBIL μmol/L</th>
<th>Diazo-TBIL μmol/L</th>
<th>Differences μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absence of β-carotenemia (n=38) &lt;2.14 μmol/L</td>
<td>0.63±0.44 (range: 0.08 to 1.63)</td>
<td>8.8±2.1 (range: 3.4 to 12.0)</td>
<td>8.9±1.9 (range: 3.4 to 12.0)</td>
<td>0.0±0.5 (range: −1.7 to 1.7)</td>
</tr>
<tr>
<td>Hyper β-carotenemia (n=5) &gt;2.14 μmol/L</td>
<td>4.71 12.0 10.3 1.7</td>
<td>2.89 17.1 12.0 5.1</td>
<td>8.16 24.0 12.0 12.0</td>
<td>4.26±2.35 16.1±4.9 10.3±1.7 5.8±4.0</td>
</tr>
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</table>

Differences=(BOX-TBIL)−(Diazo-TBIL).

Results and Discussion

Of the 42 patients with hypothyroidism, 4 patients represented β-carotenemia (>2.14 μmol/L). Contrary to the expected, TBIL concentrations in all the 42 patients measured by the BOX method were all within the normal limit (<19 μmol/L; Table 1). Elevated serum TBIL concentration (24.0 μmol/L) was observed only in the one aforementioned 59.8-y old patient with hypothyroidism who had the highest serum β-carotene concentration (8.16 μmol/L). In addition, TBIL concentrations by the diazo method in all the patients including the 59.8-y old patient were less than 19 μmol/L. However, when the differences of TBIL concentration between the BOX method and the reference diazo method were compared against the corresponding β-carotene concentrations, the differences showed an increasing trend with the increasing concentrations of β-carotene (Fig. UPLC BHE C18 column (Waters) with visible detection (472 nm). Serum specimens (or the above-mentioned β-carotene solution) were extracted with hexane, and the upper layer was evaporated under nitrogen gas to achieve dryness. The resulting residue was dissolved in aliquots of hexane and injected into the HPLC column. The reference interval for serum β-carotene concentrations was considered as 0.02–2.14 μmol/L (8).
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1. These results indicated that elevated $\beta$-carotene levels from 2.14 to 4.71 $\mu$mol/L would positively interfere with the BOX method. Although 4.26 $\mu$mol/L of $\beta$-carotene increased bilirubin levels by 5.8 $\mu$mol/L as per the BOX method, the TBIL concentrations in 4 out of 5 patients did not reach pathological levels higher than 19 $\mu$mol/L (Table 1).

Next, we conducted following in vitro experiments to identify the ingredient in the TBIL assay reagent that reacts with $\beta$-carotene as bilirubin. When a $\beta$-carotene solution was incubated at 37°C with R-1 and R-2 of the BOX method, absorbance measurements at 450 nm decreased over time (Fig. 2A). Such a decline was not observed in the absence of R-2. Thus, we filtered R-2 solution to remove BOX enzyme from R-2. The decreasing trend at 450 nm was not observed in the mixture of $\beta$-carotene solution, R-1 and the filtrate treated by Centrifree® (lacking BOX enzyme). These results indicated that the decrease of absorbance would be caused by the BOX enzyme itself and not by the buffering reagents. Because BOX method measures the intensity of the yellowish color quenched at 450 nm, the decrease in absorbance driven by $\beta$-carotene positively proved interference in the measurement of bilirubin.

On the other hand, $\beta$-carotene solution was mixed with Tris-HCl buffer, followed by the addition of the pure BOX enzyme (Sigma-Aldrich, Myrothecium verrucaria origin), and absorbance was monitored at 450 nm. A similar decreasing trend at 450 nm was observed with the use of Sigma-Aldrich BOX enzyme (Fig. 2B). In this experiment, the magnitude of decrease was proportional to the increasing concentrations of $\beta$-carotene or the increasing activity of BOX enzyme. Moreover, when R-1 and R-2 reagents of BOX method were replaced by the reagents of the VO method, absorbance at 450 nm decreased with time. On the other hand, $\beta$-carotene solution (from 0 to 16.76 $\mu$mol/L $\beta$-carotene) was diazo negative (0.09±0.14 $\mu$mol/L TBIL, on average).

Clinically, elevated serum AST and LD would suggest the presence of liver disease accompanied by elevated levels of TBIL and DBIL. However, since the patient did not show elevations of DBIL and ALT (a more specific indicative enzyme of liver damage than the AST), liver disease was ignored. In addition, since symptoms of hemolytic anemia were not observed, causes of elevated TBIL was unresolved at the patient’s first visit.

Finally, we show that the yellowness of the skin was caused by $\beta$-carotene probably driven by hypothyroidism along with elevations of CK, AST and LD originating from skeletal muscle. Patient studies and in vitro experiments strongly suggested that $\beta$-carotene could be quenched by BOX enzymatically or by vanadate chemically. Because BOX is a member of the multicopper oxidase family, we hypothesize that the copper present in the BOX enzyme oxidized $\beta$-carotene. In addition, we reconfirmed that the traditional diazo method did not react with the $\beta$-carotene in the TBIL measurement.

However, a limitation of this study is that the cases were secondary carotenemia. Effect of $\beta$-carotene in primary carotenemia (i.e., overconsumption of carotenoid-rich foods) requires further investigation.

In conclusion, an apparent elevation of TBIL (by BOX method) observed in the patient would be a result of significantly elevated $\beta$-carotene in the serum at 8.16 $\mu$mol/L.

Disclosure of state of COI

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Author contributions
SK and HI designed the research; SK, HI, MK, AT and NH performed the research and interpretation of data; MK and AT were involved in gaining ethical approval; SK and HI wrote the first draft of the manuscript; and all authors reviewed and edited the manuscript and approved the final version of the manuscript.

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