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ORIGINAL CONTRIBUTION

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Vitamin A equivalence of β-carotene in a Woman as determined by a stable isotope reference method

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Summary *Background:*Quantitative information on conversion of β-carotene to vitamin A in humans is limited.

Aim of the study: Our laboratory has developed a stable isotope method for studying the conversion of β-carotene (β-C) to vitamin A.

Methods: Two dosage levels (a pharmacological dose, 126.0 mg β-C-d_8, and a physiological dose, 6.0 mg β-C-d_8) were used 2.5 y apart in an adult female volunteer to study dose effects on the conversion of β-C to vitamin A. Blood samples were collected over 21 d. β-C and retinol were extracted from serum and isolated by high performance liquid chromatography. The retinol fraction was derivatized to a trimethylsilyl ether which was analyzed by gas chromatograph/mass spectrometry with electron capture negative chemical ionization.

*Results:*The retinol-d_4 response in the circulation peaked at 24 hours after the β-C-d_8 dose, with a higher percent enrichment after the pharmacological dose than after the physiological dose. By using retinyl acetate-d_8 as the vitamin A reference, the retinol-d_4 formed from 6 mg of β-C-d_8 (11.2 μmol) was calculated to be equivalent to 1.6 mg of retinol (i. e., 3.8 mg of β-C was equivalent to 1 mg of retinol). However, the retinol-d_4 formed from 126 mg of β-C-d_8 (235 μmol) was equivalent to 2.3 mg of retinol (i. e., 55 mg β-C was equivalent to 1 mg retinol).

Conclusion: These results provide evidence that it is feasibile to use stable isotope reference method to study retinol equivalence of β-C and that there may be a dose-dependence on bioconversion of β-carotene to retinol.

Key words β-carotene-d_8 – retinol-d_4 – humans – retinol equivalence – stable isotope – mass spectrometry

Introduction

Dietary provitamin A carotenoids can be converted to vitamin A and thus supply vitamin A to humans (1–5). β-carotene (β-C) is a prominent provitamin A carotenoid in human diets, and the retinol equivalence of dietary β-C has been defined as 6 μg of β-C being equal to 1 μg of retinol (6). This conversion factor was mainly based on depletion-repletion studies carried out in a few male subjects (6, 7).

However, in animal studies, it was found that the bioconversion of β-C to vitamin A is decreased at increasing doses of β-C (8). In humans, chronic high doses of β-C for the prevention of photodermatoses have not resulted in vitamin A toxicity, which implies that the extent of bioconversion of dietary β-C to vitamin A is decreased when the intake of β-C is increased to pharmacological levels.

Our laboratory has developed a stable isotope method for studying the conversion of β-C to vitamin A (9). To evaluate the vitamin A equivalence of β-C in well-nourished humans with minimal invasiveness, we used stable

isotope labeled β-C-d_8 to study formation of retinol-d_4. Further, we used vitamin A-d_8 as a vitamin A isotope reference to determine quantitatively the retinol equivalence of the β-C-d_8 dose. Two levels of doses (a pharmacological dose, 126.0 mg β-C-d_8, and a physiological dose, 6.0 mg β-C-d_8) were used to study the dose effect on the conversion of β-C to vitamin A.

Material and methods

Deuterated β-C and retinyl acetate

Crystalline all-trans β-C-d_8 (11, 11', 19, 19, 19, 19', 19', 19'–$^2H_8$-β-C, 82 % in the all-trans form, 8 % in the 13-cis form, 4.2 % in the 9-cis form and 3.4 % in the 15-cis form) in a sealed amber ampoule was provided by BASF (Ludwigshafen, Germany). The purity of β-C-d_8 was checked by HPLC and was 97.5 % spectroscopically pure, but it contained β-C-d_7 (15.7 %), β-C-d_0 (2.9 %) and β-C-d_6 (0.3 %) as measured by Atmospheric Pressure Chemical Ionization-Mass Spectrometer (10). The retinyl acetate-d_8 (10, 14, 19, 19, 19, 20, 20, 20–$^2H_8$-retinyl acetate) was synthesized in this laboratory as described previously (9).

Sample preparation

Subject and blood sample collection

Blood sampling followed the procedures set by the Human Investigation Committee at Tufts University and the New England Medical Center. After an overnight fast, an adult female volunteer (Body Mass Index = 25.2–25.6 kg/m^2 over the 2.5 y study period), consumed gelatin capsules containing a total of 126.0 mg of β-C-d_8 (235 μmol) in 2 g corn oil with a standardized high fat breakfast (30 g fat from two plain bagels and 40 g almond butter). Serum samples were collected at 0, 3, and 6 h and at 1, 2, 4, 8, 14, and 21 d after the β-C-d_8 administration, and were stored at –70 °C. For 21 d, the subject consumed regular meals. Two and a half years later, the same volunteer consumed 6.0 mg of β-C-d_8 (11.2 μmol) in 0.5 g corn oil with the same standardized high fat breakfast and donated blood samples as described above. Retinyl acetate-d_8 [10, 14, 19, 19, 19, 20, 20, 20–$^2H_8$-retinyl acetate, 9 mg (30.6 μmol)] was dissolved in 0.5 g corn oil and was taken by the same subject between the two doses of β-C-d_8 (2 years after the initial 126.0 mg β-C-d_8 dose), and blood samples were collected as above plus an additional blood sample at 9 hours. Blood samples were kept at room temperature for half an hour and then centrifuged with Sure-sep II (Organon Teknika Corp., Durham, NC) at 4 °C and 800*g* for 15 min. Serum was stored at –70 °C until processed.

Extraction and separation of retinol in serum

Two ml of chloroform/methanol (2:1 in volume) was added to a 200 μL serum sample. The mixture was vortexed and centrifuged for 10 min at 4 °C and at 800*g*. The chloroform layer was collected. Hexane (2 mL) was added to the aqueous layer to re-extract fat soluble nutrients. The hexane layer was combined with the chloroform layer and evaporated under nitrogen on a N-EVAP evaporator (Organomation Associates, Inc., South Berlin, MA). The residue was dissolved in 80 μL of ethanol and 50 μL was injected onto an HPLC equipped with a C18 column (Perkin-Elmer Inc., Norwalk, CT) (11). The gradient solvent procedure was as follows: 100 % solvent A (acetonitrile/tetrahydrofuran/water, 50/20/30, v/v/v, 10 g/L ammonium acetate in water) was used for 6 min followed by an 8-min linear gradient to 50 % solvent B (acetonitrile/ tetrahydrofuran/water, 50/44/6, v/v/v, 10 g/L ammonium acetate in water), a 5 min hold at 50 % solvent B, a 2 min gradient to 100 % solvent B, followed by an 8 min hold at 100 % B, and a 1 min gradient to 100 % A (12).

Sample analysis

HPLC analysis of serum samples

Concentrations of β-C and retinol in an 100 μL aliquot of serum were measured by an HPLC equipped with a Pecosphere–3 C18 column (Perkin-Elmer, Norwalk, CT) and a Waters 994 Programmable Photodiode Array Detector (Milford, MA) with the wavelength set at 450 nm for carotenoids and 340 nm for retinoids (11). The concentrations of retinol in serum and the percentage isotopic enrichment of retinol-d_4 derived from β-C-d_8 or retinol-d_8 derived from retinyl acetate-d_8 were used to calculate the molar enrichment of retinol-d_4 or retinol-d_8 in the circulation.

GC/MS analysis of retinol in human serum

We have improved our retinol GC/MS method by using a convenient derivatization procedure (9, 10). The derivatized retinol was analyzed by electron capture negative chemical ionization GC/MS. The percentage enrichment of retinol-d_4 derived from β-C-d_8 was calculated by integrating the peak area under the re-constructed mass chromatogram of the negative ions at m/z 271 (d_3), 272 (d_4 + ^{13}C-d_3, and 273 (^{13}C-d_4) divided by the total area response of labeled and unlabeled retinol ions. The percentage enrichment of retinol-d_8 derived from retinyl acetate-d_8 was calculated by integrating the peak area under the re-constructed mass chromatogram of the negative ions at m/z 274 (d_6), 275 (d_7), 276 (d_8), 277 (d_8 + ^{13}C-d_8), and 278 ($^{13}C_2$-d_8) divided by the total area response of labeled and

unlabeled retinol fragment ions. The percentage enrichment measured by GC/MS and the concentration of retinol in the serum were used to calculate the concentration of labeled retinol in the circulation.

Areas under the serum labeled retinol response curves

Areas under the 21 day serum labeled retinol response curves (arbitrary scale in nmole-day unit) after the β-C-d_8 doses and after the retinyl acetate-d_8 dose were calculated by using Kalaidagraph (Synergy Software, Reading, PA).

Results

The volunteer had normal vitamin A nutrition with a serum retinol concentration ranging from 1.49 μmol/L to 1.52 μmol/L over the 2.5 y experimental period. Serum concentrations of β-C after the 6 mg β-C dose did not change during the experimental period (Fig. 1). However, serum concentration of β-C after 126 mg β-C dose was approximately three times the baseline concentration of β-C (from 0.42 μmol/L at the baseline to 1.29 μmol/L at 24 h after the dose) (Fig. 1). The retinol-d_4 responses in the circulation peaked at 24 h after the β-C-d_8 dose with a higher molar enrichment after the pharmacological dose than after the physiological dose (Fig. 2). Two weeks after the β-C-d_8 administration, retinol-d_4 in serum reached an almost identical enrichment in the body at both the pharmacologic and physiologic doses (Fig. 2). The area under the 21 day serum retinol-d_4 concentration curve after the pharmacological dose of β-C-d_8 (126.0 mg) was 460 units (nmol · day) while after the physiological dose of β-C-d_8 (6.0 mg) it was 315 units (nmol · day), as shown in Table 1.

The serum retinol-d_8 response to the retinyl acetate-d_8 dose showed a maximum at 24 h after the dose (Fig. 2). The area under the 21 day serum retinol-d_8 concentration curve after the retinyl acetate-d_8 dose was 1,564 nmol · day (Table 1).

By using retinyl acetate-d_8 as a vitamin A reference, the retinol-d_4 formed from 126 mg of β-C-d_8 (235 μmol) was equivalent to 2.3 mg of retinol. Therefore, following a pharmacological dose, 55 mg of β-C was equivalent to 1 mg of retinol. The retinol-d_4 formed from 6 mg of β-C-d_8

Table 1 Vitamin A equivalence of β-carotene-d_8 determined in a woman using retinyl acetate-d_8 as an isotope reference1

Dose	Area under retinol response curve (nmol · day)	Retinol equivalence[2] (x 10^3)	Conversion factor[3]
b-carotene-d_8 126.0 mg	460	2.3	55 to 1
β-carotene-d_8 6.0 mg	315	1.6	3.8 to 1
Retinyl acetate-d_8 9.0 mg	1,564	7.7	1.1 to 1

[1] Values are obtained from the area under the serum response curves of the percent enrichment of retinol-d_4 in sera collected at 0, 3, and 6 hours and at 1, 2, 4, 8, 14, and 21 days following 126 mg or 6 mg β-carotene-d_8 as well as percent enrichment of retinol-d_8 in sera collected at 0, 3, 6 and 9 hours and at 1, 2, 4, 8, 14, and 21 days following a 9.0 mg retinyl acetate-d_8 dose.

[2] Retinol equivalent = 1 μg retinol. The values in this column were calculated based on the area under the labeled retinol response curves (AUC). Using AUC 1564 from 9.0 mg of retinyl acetate-d_8 dose as a reference as being equal to 7.7 x 10^3 RE, 126.0 mg β-C provided 460/1564 x 7.7 x 10^3 = 2.3 x 10^3 RE and 6.0 mg β-C provided 315 / 1564 x 7.7 x 10^3 = 1.6 x 10^3 RE.

[3] Conversion factors were calculated as Dose (mg) / RE (10^3), e. g.,

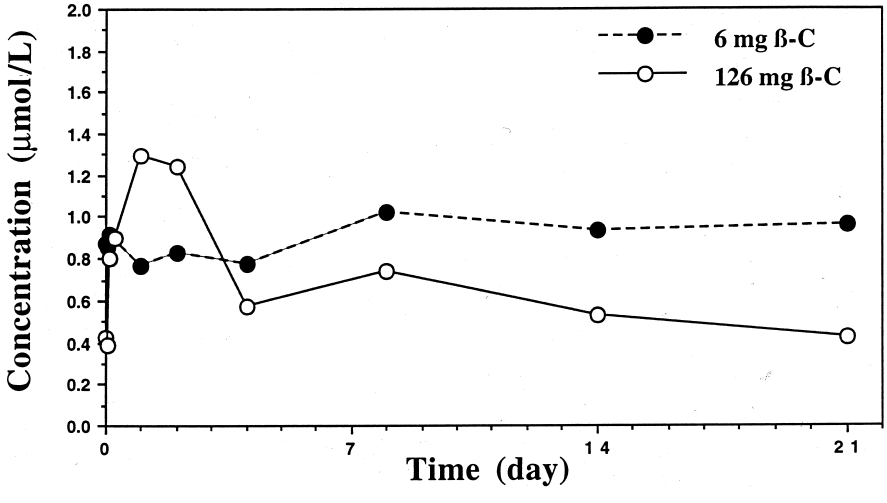

Fig. 1 Serum concentration of β-carotene measured following administration of a 6 mg (solid circle) or 126 mg (open circle) β-carotene-d_8 dose.

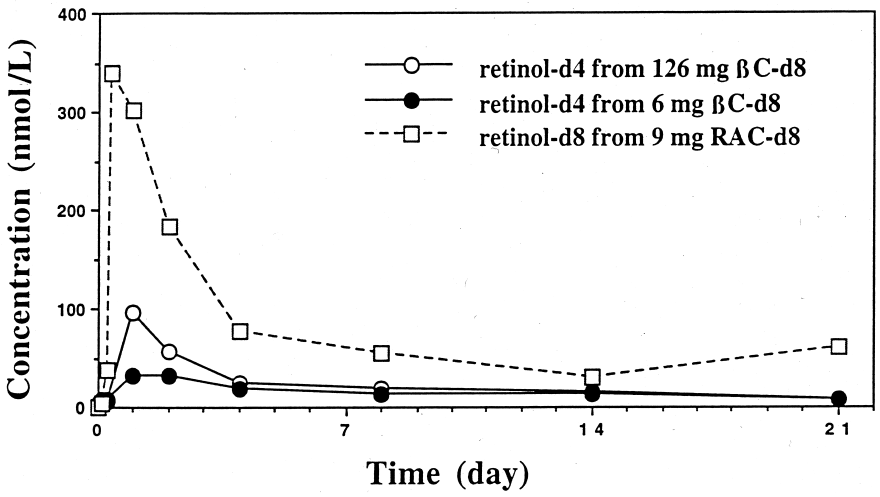

Fig. 2 The enrichment of retinol-d_4 in the serum collected at 0, 3, and 6 hours and at 1, 2, 4, 8, 14, and 21 days following 6 mg (solid circle) or 126 mg (open circle) β-carotene-d_8 as well as enrichment of retinol-d_8 in the serum collected at 0, 3, 6 and 9 hours and at 1, 2, 4, 8, 14, and 21 days following a 9.0 mg retinyl acetate-d_8 (open square) dose.

(11.2 μmole) was equivalent to 1.6 mg of retinol. Therefore, following a physiological dose, 3.8 mg of β-C was equivalent to 1 mg of retinol.

Discussion

The purpose of this paper is to rapidly communicate the quantitative evaluation of the bioconversion of β-C to vitamin A in humans, since there are limited data on this issue. The kinetic responses of labeled retinol formed from the precursor β-C or from a known amount of vitamin A were compared to determine the vitamin A value of the β-C dose in a healthy female subject with normal vitamin A nutrition and serum concentration of β-C. We show that it is possible to obtain vitamin A value of provitamin A carotenoids without using depletion-repletion procedures in a well-nourished population.

In 1974, Sauberlich et al. depleted 8 male subjects and repleted 3 of them with β-C and 5 of them with retinyl acetate. They calculated that the daily intake of 2.4 mg β-C was equivalent to daily intake of 1.2 mg vitamin A. Compared with the results obtained by Sauberlich et al. (2 μg of β-C was equivalent to 1 μg of retinol), our results show that a 6 mg β-C dose was less effective, that is, 3.8 μg of β-C was equivalent to 1 μg of retinol. This may due to the difference in vitamin A status of the volunteers in the two studies. In our study, the serum vitamin A level was 1.6 μmol/L while in Sauberlich's study, after 103 days of vitamin A depletion, the plasma vitamin A levels of most of the subjects was < 0.35 μmol/L. It has been found that the activity of intestinal β-C cleavage enzyme in vitamin A sufficient rats was 50 % of that in vitamin A deficient rats (13). Furthermore, our study used 6 mg β-C and their study used doses < 2.4 mg β-C. Even dose variation within this range may affect the conversion rate of carotenoids to vitamin A.

The extent of bioconversion of dietary β-C depends on many factors, such as composition of the overall diet, gastrointestinal absorption, vitamin A status, and the amount of β-C in a single dose. By using the same subject with normal *and stable* vitamin A status and giving the labeled doses with the standardized meals, we studied the bioconversion of β-C to vitamin A at two different β-C dosage levels. Even though the purpose of this study was to evaluate the feasibility of using an isotope reference method and only one female subject was tested, it was clear that the efficiency of bioconversion of β-C to vitamin A was dose dependent. β-C is absorbed in the small intestine through a simple diffusion process, which is affected by the β-C concentration gradient between the luminal pool and the intracellular pool (14). A difference in blood response to two doses of β-C (12 mg and 30 mg) in 15 human subjects was observed by Brown et al. (15). Their study showed a decreased blood response efficacy with an increasing β-C dose, that is, the ratio of the two doses was 2.5, whereas the ratio of the blood AUC (area under the β-C concentration curve) was only 1.9. Moreover, supplementation with a high dose of β-C may exceed the limited capacity of small intestinal β-C cleavage enzyme(s) to digest a large dose of β-C. Until now, post-absorption conversion of β-C has hardly been investigated. However, because a 21 times the β-C dose produced a 1,5 times the vitamin A, our results demonstrate that more than 6 mg of β-C can be converted to vitamin A (i. e., 6 mg is not a "saturating" dose). Using this isotope reference method, a full investigation of vitamin A equivalence at various β-C dose levels, such as 1.5, 3.0, 12.0, and 24.0 mg of β-C, will enable us in the future to determine the linearity and saturation level of ingested β-C for conversion to vitamin A.

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