A novel extrinsic reference method for assessing the vitamin A value of plant foods[1–3]

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ABSTRACT

Background: The amounts of vitamin A that are metabolically derived from specific carotene-containing foods are largely unknown.

Objective: We sought to develop an improved method for estimating the metabolic vitamin A potential of provitamin A carotenoids by using [^{2}H$_4$]retinyl acetate (d$_4$-RA) as an extrinsic reference standard.

Design: Healthy subjects consumed a standardized test meal containing 6 mg β-carotene as either raw carrot or spinach, either 20 or 1 g added fat, and 6.0 μmol d$_4$-RA. Concentrations of unlabeled (d$_0$) retinyl esters (RE), labeled (d$_4$) RE, and carotenoids in the plasma triacylglycerol-rich lipoprotein fraction ($d < 1.006$ kg/L) were determined in serial blood samples with HPLC and gas chromatography–mass spectrometry. Baseline-corrected areas under the curve for d$_0$-RE, d$_4$-RE, and carotenoids were calculated, and the masses of absorbed d$_0$-retinol and carotenes were estimated assuming 80% absorption of the d$_4$-RA reference dose.

Results: In trials with ample (20 g) fat ($n = 6$), 7 ± 4% of the 6 mg β-carotene ingested was taken up as β-carotene plus RE with 0.3 ± 0.1 mg as retinol. Test meals without carotenes yielded no β-carotene or d$_0$-RE response and there was no effect of treatment (either fat amount or vegetable, $n = 6$) on the mean d$_4$-RE area under the curve. The lower-than-expected vitamin A yields were attributed to poor intestinal uptake rather than to low conversion of β-carotene to RE.

Conclusion: The triacylglycerol-rich lipoprotein and d$_4$-RA method, which controls for variation in chylomicron kinetics in vivo and RE recovery during analysis, is useful for obtaining quantitative estimates of the vitamin A potential of single meals. *Am J Clin Nutr* 2001;74:348–55.

INTRODUCTION

Provitamin A carotenes, particularly β-carotene from plant foods, are a significant source of vitamin A for many populations, including the US population (1). However, methods are lacking for determining the amount of vitamin A actually assimilated from foods containing these carotenes. In the mid-1990s, findings from field studies suggested that the ability of some plant foods to improve vitamin A status was less than expected; this stimulated renewed interest in clarifying the vitamin A value of such foods (2, 3).

van Vliet et al (4) showed that the plasma triacylglycerol-rich lipoprotein fraction (TRL, $d < 1.006$ kg/L) may be useful in the assessment of β-carotene uptake and intestinal metabolism to retinyl esters (RE) after an oral dose of β-carotene. RE and β-carotene responses were not evident in whole plasma but were quantifiable in the TRL fraction after a 15-mg dose of β-carotene. The TRL-response approach was used in a limited number of studies to assess carotenoid bioavailablity from tangerine concentrate (5), tomatoes and tomato paste (6), water-dispersible β-carotene beadlets (4, 7), and β-carotene supplements (8). van den Berg and van Vliet (9) reported that carotenoid and RE responses were substantially lower after consumption of vegetables, including carrots and spinach, than after ingestion of carotenoid concentrates.

An advantage of the TRL approach is its use of the chylomicron-rich fraction, which contains newly absorbed lipids (including β-carotene and RE) from a recent test meal. Secretion of RE and β-carotene by the small intestine into the general circulation occurs exclusively via chylomicron transport. However, interpretation of TRL data was limited by the lack of means to control for interindividual variation in in vivo chylomicron clearance kinetics or variation in chylomicron recovery during TRL preparation and analysis. Consequently, use of this approach generally was restricted to comparative (between-treatment) studies because it does not directly measure the mass of RE or β-carotene absorbed.

Here we describe an adaptation of the TRL-response model that involves co-administration of a small quantity of [10,19,19,19-^{2}H]retinyl acetate (d$_4$-RA) in a maximally bioavailable form (oil solution). This extrinsic reference dose controls for variation in chylomicron kinetics in vivo and for RE recovery during TRL

preparation and analysis and also acts as a reference with which to estimate the mass of unlabeled vitamin A derived from intestinal cleavage of provitamin A carotenes taken up from a simple test meal.

SUBJECTS AND METHODS

Subjects

All procedures involving human subjects were approved by the Cornell Committee on Human Subjects Research and each subject provided written, informed consent. Three apparently healthy, nonsmoking adults (1 woman and 2 men, aged 25–35 y) were recruited from the Cornell community. The subjects were screened by conducting a health history interview and by clinical evaluation that revealed no evidence of chronic gastrointestinal disease, liver disease, or hyperlipidemia. Body mass index values (in kg/m^2) for all subjects ranged from 21 to 23. Subjects reported no use of vitamin A or β-carotene supplements within the past 2 mo or longer. Fasting plasma retinol and β-carotene concentrations were, respectively, 1.91 and 0.59 μmol/L for subject 1, 1.32 and 0.07 μmol/L for subject 2, and 1.81 and 0.28 μmol/L for subject 3.

Subjects were instructed to avoid foods that contain carotenoids or are rich in vitamin A for 24 h before each trial and they completed food diaries during these time periods. Subjects fasted for 12 h before coming to the laboratory in the morning to provide a baseline blood sample. All foods eaten during the test day were consumed on-site in the presence of at least one investigator to verify complete consumption of the test meal and the absence of other sources of carotenoids or vitamin A.

Treatments

A single-dose, pharmacokinetic approach was used. Four different test meals were administered to each of the 3 subjects, in random order, 2–6 wk apart. The meals were *1)* raw carrot + 20 g fat, *2)* raw spinach + 20 g fat, *3)* raw carrot + 1 g fat, and *4)* raw spinach + 1 g fat. Trimmed spinach and baby carrots were purchased from local supermarkets 1 d before consumption. Spinach leaves were washed, trimmed, and blotted dry before serving. Identical samples were frozen at −80°C for later analysis. Carrots were consumed as purchased. The fiber contents of the doses of spinach and carrots were calculated as 3.3 g and 1.1 g, respectively (10). The fat source was high-oleate safflower oil containing 74% monounsaturated fat, 17% polyunsaturated fat, and 9% saturated fat (Pure Pressed Spectrum Naturals Inc, Petaluma, CA) and was purchased at a local supermarket.

All test meals included 6.0 μmol (2 mg) d$_4$-RA dissolved in 1 g safflower oil. The d$_4$-RA was purchased from Cambridge Isotope Laboratories (Andover, MA) and was verified in our laboratory to be of >98% chemical purity by analytic HPLC and of 99.5% isotopic purity by gas chromatography–mass spectrometry.

Stock solutions of d$_4$-RA in safflower oil were prepared by first dissolving 20 mg d$_4$-RA in 2 mL absolute ethanol; the concentration was measured spectrophotometrically at 325 nm. Two milliliters of this solution was slowly added to 10 g safflower oil at 35°C while stirring gently under a N$_2$ stream for ≈1 h to completely remove the ethanol. The absence of ethanol was verified gravimetrically. The final stock solution of 2 mg d$_4$-RA/g safflower oil was stored at 4°C under argon in the dark until use. For each trial, 1 g of the d$_4$-RA and oil solution was dispersed in a blended drink containing 70 mL diluted pear juice (filtered through cheesecloth and diluted 1:1 with water to reduce the solids content), 30.0 g banana (to stabilize the emulsion and provide flavor), and the appropriate amount of additional safflower oil (0 or 19 g, depending on the treatment). For all test meals, the drink container and blender attachment were rinsed with an additional 100 mL diluted pear juice, which was consumed by the subject to ensure complete administration of the dose. Analysis of the residual indicated that >98% of the dose of d$_4$-RA was ingested.

The test meal was administered at 0830 after collection of a baseline blood sample. Subjects were given 100 mL water 30 min later and were allowed free access to water after 60 min. A standardized midday meal that was carotenoid-free and very low in vitamin A was provided 3.25 h postdose. This midday meal contained the same amount of fat as the test meal. The high-fat midday meal consisted of noodle soup, one plain bagel, and 15 g regular cream cheese. The low-fat midday meal consisted of 2 plain bagels and 11.25 g nonfat cream cheese. The midday meal provided ≈57 μg retinol in all trials (10). All midday meals also included 240 mL apple juice and either one green-skinned apple, a banana, or green grapes.

An additional trial was conducted in one of the subjects, who consumed a low-fat (1 g fat) carrot test meal followed by a high-fat (20 g fat) midday meal 3.5 h later. We also conducted 2 additional control trials in which the test meal contained either 20 g safflower oil only (no carotenoids or d$_4$-RA; administered to subjects 2 and 3) or 20 g safflower oil containing 2 mg d$_4$-RA (no carotenoids; administered to subject 2). In both control meals, subjects consumed one-half of a plain bagel, which provided bulk with minimal fiber (<0.2 g).

Determination of test meal carotenoid contents

The carotenoid contents of the spinach and carrots that the subjects consumed were determined by using the method of Khachik and Beecher (11), with slight modifications. Solvents were HPLC grade and were purchased from Fisher Scientific (Fair Lawn, NJ). Magnesium carbonate (10% of weight of food) was added to food samples (12–20 g), which were homogenized in a Waring blender (Dynamics Corp of America, New Hartford, CT) with 150 mL tetrahydrofuran containing 5 mg ethyl β-apo-8′-carotenoate (Fluka, Milwaukee) as an internal standard and 0.01% butylated hydroxytoluene. The tetrahydrofuran extract was filtered through no. 1 Whatman filter paper (Whatman International Ltd, Maidstone, United Kingdom) and the remaining plant residue was reextracted with 100 mL tetrahydrofuran until it was colorless. The total tetrahydrofuran extract was concentrated to 80–100 mL on a rotary evaporator at 30°C and then partitioned between petroleum ether and saturated sodium chloride solution. The petroleum ether phase was dried over magnesium sulfate and filtered through no. 2V Whatman filter paper (Whatman International Ltd); a sample was then analyzed by reversed-phase HPLC as described below. Actual carotenoid doses were determined for each test meal. The average β-carotene dose was 5.6 ± 0.8 mg for carrot meals and 6.2 ± 0.8 mg for spinach meals. On average, the carrot meals also supplied 3.1 ± 1.1 mg α-carotene and the spinach meals supplied 5.9 ± 2.5 mg lutein.

Sample collection and preparation

Blood samples were drawn by venipuncture into EDTA-coated evacuated tubes by a trained phlebotomist. Six time points were sampled in each trial: 0 (baseline, fasting), 2, 3.5,

4.5, 5.5, and 7 h for the high-fat treatments and 0, 3, 4.5, 5.5, 7, and 8.5 h for the low-fat treatments. Plasma was separated by low-speed centrifugation (400 × *g* for 5 min at 20 °C) and stored at 2–4 °C until ultracentrifugation later the same day. The *d* < 1.006 kg/L plasma fraction was obtained by standard ultracentrifugation techniques (12). Briefly, 4 mL plasma was added to 13 × 64-mm Beckman Bell-Top Quick-Seal centrifuge tubes (Beckman Instruments Inc, Palo Alto, CA) and overlaid with 2 mL 0.195-mol NaCl/L solution (*d* = 1.006 kg/L) containing 2.69 × 10^{-4} mol EDTA-Na$_2$/L and 0.001 mol NaOH/L. Tubes were prepared in duplicate or triplicate for each time point and were spun at 180 000 × g_{av} for 14 h at 4 °C in a Beckman 50.3 titanium fixed-angle rotor and Beckman model L5-50 ultracentrifuge (Beckman Instruments Inc). These conditions resulted in a clear separation between the floating TRL fraction and the denser lipoprotein fractions present in the infranatant fluid. The *d* < 1.006 kg/L fractions were collected by using a tube slicer for preparative ultracentrifuge tubes to remove the top 1.5 cm of the tube. The knife and centrifuge tube top were rinsed with 2 mL *d* = 1.006 kg/L salt solution to facilitate complete collection of the TRL fraction. TRL fractions were flushed with N$_2$ or argon and stored at −80 °C until extraction and analysis.

The total lipids contained in the TRL fraction were extracted as described previously (13). Protein was precipitated with 1 volume ethanol containing 2.5 μg δ-tocopherol (Sigma Chemical Co, St Louis) and 0.1 μg echinenone (Fluka) as internal standards and 0.02% (wt:vol) butylated hydroxytoluene. The sample was extracted twice with 3 volumes of hexane. The dried extracts were redissolved in 1.6 mL 1% potassium hydroxide in ethanol and were then saponified at 60 °C under N$_2$ for 30 min to hydrolyze retinyl esters to retinol and remove triacylglycerol. Cooled samples were extracted twice with 2 mL hexane after the addition of 1.2 mL deionized water. The hexane extract was washed with 2 mL water, dried under N$_2$, and redissolved in 48 μL dimethylformamide plus 250 μL HPLC mobile phase (described below) for HPLC analysis.

Analysis of carotenoids and vitamin A in the TRL fraction

Concentrations of *all-trans*-β-carotene, α-carotene, lutein, and RE-derived retinol (d$_0$- plus d$_4$-retinol) extracted from the TRL fraction were determined by using a modification of the HPLC method of Thurnham et al (14). δ-Tocopherol was used as the internal standard for retinol and lutein, 2 of the more polar components, and echinenone was used as the internal standard for α-carotene and *all-trans*-β-carotene. Both internal standards were undetectable in native TRL fractions. A 15-cm Spherisorb ODS-2 column (Phase Separations Inc, Norwalk, CT) was used with a mobile phase of methanol:acetonitrile:chloroform (47:47:6, by vol), 0.05% triethylamine, and 0.05 mol ammonium acetate/L. The HPLC system consisted of a Waters 996 Photodiode Array Detector, 717 Plus Autosampler and MILLENIUM software, version 2.1 (Waters, Milford, MA) along with a Hewlett-Packard series 1050 HPLC pump (Hewlett-Packard, Wilmington, DE). Retention times (RT) of retinol, lutein, δ-tocopherol, echinenone, α-carotene, and *all-trans*-β-carotene were 2.7, 2.9, 5.6, 9.5, 21.9, and 24.1 min, respectively, at a flow rate of 1 mL/min. Carotenes and retinol were identified by RT and ultraviolet-visible spectra and were quantified by reference to standard curves of response values. The spectrum of the lutein peak corresponded to that of a lutein standard, but at least partial coelution of lutein with zeaxanthin may have occurred with this method. Response was

taken as the height of the retinol or carotene peak divided by the height of the internal standard peak. Analyses of whole plasma retinol and β-carotene concentrations at baseline were also performed by using HPLC with this method.

The RE-derived retinol fraction was collected from HPLC, dried under N$_2$, and silylated with 50 μL *N,O*-bis(trimethylsilyl) trifluoroacetimide (TMS) and 100 μL pyridine (Pierce, Rockford, IL) at room temperature in the dark for 1 h. The relative proportions of d$_0$-retinol-TMS and d$_4$-retinol-TMS were determined by using gas chromatography–mass spectroscopy. We used a Hewlett Packard HP 6890 series gas chromatograph (Hewlett-Packard, Palo Alto, CA) coupled to a Hewlett-Packard model 5972 mass selective detector (Hewlett-Packard, Wilmington, DE) fitted with a 30-m DB-1 column (internal diameter, 0.25 mm; film thickness, 0.25 μm; J & W Scientific, Folsom, CA). The gas chromatograph was operated at an inlet temperature of 300 °C, head pressure of 238.5 kPa, and split ratio of 2:1. Gas chromatograph oven conditions were as follows: initial temperature of 220 °C (2 min hold), temperature ramp of 15 °C/min up to 260 °C (no hold), and temperature ramp of 25 °C/min up to 280 °C (10 min hold). The linear velocity of the helium carrier gas was 68 cm/min and the total run time was 15.6 min. Applied electron multiplier voltage was 2500 V and output was monitored at a mass-to-charge ratio (*m/z*) of 358.4 for d$_0$-retinol-TMS (RT = 5.22 min) and at *m/z* 362.4 for d$_4$-retinol-TMS (RT = 5.21 min).

We also determined the proportion of total plasma RE (d$_0$- plus d$_4$-RE) that was recovered in the TRL fraction after ultracentrifugation. To determine this proportion, RE in the plasma infranatant fluid (plasma minus TRL) needed to be completely separated from unesterified retinol, which in whole plasma is present in excess relative to RE. A method was developed by using a model TRL lipid mixture containing 3 mg safflower oil, 3 mg cholesterol, and 3 mg cholesterol oleate (Sigma Chemical Co) dissolved in 4 mL 95:5 (by vol) hexane:methyl *tert*-butyl ether (MTBE). To this solution was added either 9.8 nmol retinyl palmitate (solution A) or 18 nmol retinol (solution B). The optical densities of solutions A and B were measured spectrophotometrically at 325 nm before and after elution from a Sep-Pak silica cartridge (Millipore; Waters) with hexane:MTBE (95:5 by vol). Under these conditions, retinol was completely retained and retinyl palmitate was completely eluted. The cartridge was then eluted with 8 mL hexane:isopropanol (90:10 by vol) to recover the bound retinol, as verified by spectrophotometry. A subset of 5 plasma infranatant fractions obtained at the time of maximal TRL RE concentration (usually 4.5–5.5 h postdose) were deproteinated and extracted with hexanes as described for TRL. The extracts were dried, dissolved in 4 mL hexane:MTBE (95:5), and subjected to Sep-Pak fractionation as described above and the retinol and retinyl palmitate (saponified) fractions were quantitated by analytic HPLC as described above.

Calculations

The area (molar) ratio of d$_0$-retinol-TMS to d$_4$-retinol-TMS, determined by gas chromatography–mass spectroscopy, was multiplied by the concentration of total TRL RE, as determined by HPLC, to yield the concentrations of d$_0$- and d$_4$-RE in the TRL at each time point. Baseline-corrected plots of concentration versus time were generated for d$_0$-RE, d$_4$-RE, β-carotene, α-carotene, and lutein. Area-under-the-curve (AUC) values were then determined by surface-area calculations using the experimentally determined coordinates of the curve. To estimate the

mass of retinol or carotenoid assimilated from the test meal, an absorption efficiency of 80% for the 6.0 μmol d_4-RA reference dose was assumed, ie, the AUC for d_4-RE was set equivalent to 4.8 μmol RE. The amount of d_0-RE derived from the provitamin A carotenoids in the test meal was then calculated by dividing the AUC of d_0-RE by that of d_4-RE and multiplying this quotient by 4.8 μmol. The same approach was used to estimate the mass of carotenoid (β-carotene, α-carotene, or lutein) absorbed intact.

The mass of β-carotene absorbed intact was expressed as a percentage of the β-carotene dose. The total amount of β-carotene taken up from the test meal (intestinal extraction), as the combination of intact β-carotene plus d_0-RE, was also estimated. To do this, we summed the moles of β-carotene absorbed intact plus one-half the moles of RE absorbed (ie, assuming a 1:2 stoichiometry of conversion of β-carotene to RE). This value was expressed relative to the moles of β-carotene consumed in the test meal. For the carrot trials, we estimated the potential contribution of α-carotene to d_0-RE by using the molar ratio of β-carotene to α-carotene in carrots and assuming that α-carotene has one-half the molar retinol value of β-carotene. This estimate of α-carotene-derived d_0-RE was subtracted from the total moles of RE absorbed to give the amount of RE or retinol derived only from absorbed β-carotene.

Statistical analyses

Each of the 3 subjects participated in all 4 treatments (2 vegetables, each tested with 2 amounts of added fat). To determine whether the d_4-RE response (AUC) varied systematically by treatment (vegetable or amount of fat), the following analyses were conducted. Repeated-measures analysis of variance was used to test for the effects of fat amount and vegetable and the interaction between fat amount and vegetable. Because no interaction was evident, a paired t test (two-tailed) was conducted to compare the mean d_0-RE AUC for all high-fat vegetable treatments ($n = 6$) with the corresponding mean for all low-fat vegetable treatments ($n = 6$). A similar test was used to compare the mean d_0-RE AUC between high-fat carrot ($n = 3$) and high-fat spinach ($n = 3$) treatments. Values from the low-fat treatments were omitted from this latter analysis because of perturbation in the absorption profiles, as described below. Log transformation of the data did not change the outcomes. All analyses were performed with SPSS for WINDOWS (version 8.0; SPSS Inc, Chicago). $P < 0.05$ was considered statistically significant.

RESULTS

TRL-response profiles for d_4-RE, d_0-RE, and β-carotene

Postprandial concentrations of retinyl esters and carotenes in the $d < 1.006$ kg/L plasma fraction after a test meal containing ≈ 6 mg β-carotene as carrot or spinach plus 2 mg d_4-RA are illustrated in **Figure 1**. Concentrations of d_4-RE, d_0-RE, and β-carotene peaked between 4.5 and 5.5 h in all high-fat trials, regardless of β-carotene source, and returned to near baseline by 7 h, when the trials were terminated. Similarly, α-carotene and lutein peaked at 4.5–5.5 h for the high-fat carrot and spinach trials, respectively. In contrast, low-fat test meals resulted in attenuated and prolonged absorption profiles, with TRL concentrations of d_0-RE remaining elevated above baseline values at 8.5 h, when these trials were terminated.

The d_4-RE responses in the TRL fraction for all 12 vegetable trials are shown in **Figure 2**. Individual d_4-RE AUC values

ranged from 794 to 2099 ($\bar{x}$: 1488 ± 397) nmol·h/L. Within each subject, d_4-RE values varied between the 4 trials by as much as 1077–2099 nmol·h/L. An example of variation in d_4-RE response curves within a single subject is shown in Figure 1, A and B. For this subject, the AUC for d_4-RE was lower after the low-fat meal than after the high-fat meal. The AUC for d_4-RE varied much less under different fat conditions for a second subject (Figure 1, C and D). In a third subject (not shown), the AUC for d_4-RE was greater after a low-fat meal than after a high-fat meal. There was no significant difference between the mean d_4-RE AUC for high-fat meals ($\bar{x}$: 1539 ± 476 nmol·h/L; $n = 6$) and low-fat meals ($\bar{x}$: 1399 ± 360 nmol·h/L; $n = 6$). Similarly, no significant difference in mean d_4-RE AUC was found between the different food sources of β-carotene.

Estimated masses of vitamin A and β-carotene derived from the test meals

The estimated masses of unlabeled retinol (as RE) and β-carotene secreted into the bloodstream after consumption of the test meals were calculated by reference to the d_4-RA AUC, which was assumed to be equivalent to 80% of the d_4-RA dose. Because the β-carotene dose varied slightly from trial to trial, these estimates were normalized to a 6-mg dose of β-carotene for comparison purposes (**Figure 3**, A-D). In the high-fat vegetable trials, the amount of retinol estimated to be derived from 6 mg β-carotene ranged from 0.14 to 0.53 mg ($\bar{x}$: 0.30 ± 0.13 mg). This amount was particularly consistent for the high-fat carrot treatment ($\bar{x}$: 0.28 ± 0.01 mg). This consistency was not evident in the raw d_0-RE AUC values, which ranged from 240 to 337 nmol·h/L, but was apparent only when these values were normalized to the corresponding d_4-RE AUC values.

The masses of retinol and β-carotene derived from the low-fat test meals (Figure 3, B and D) were generally lower than the corresponding masses derived from the high-fat test meals in the same subjects fed an equivalent amount of β-carotene. However, because the entire absorption episode was apparently not captured within the 8.5-h data collection period, the values for retinol and β-carotene absorbed under low-fat conditions may be underestimated.

The ratio of the mass of retinol absorbed (as RE) to the mass of intact β-carotene absorbed was not constant, either between or within subjects, and ranged from 5.8:1 to 0.7:1 in the 6 high-fat trials. With only one exception (Figure 3A, subject 2), the mass of absorbed retinol was greater than that of intact β-carotene.

Subjects restricted their carotenoid intake for 24 h before each test meal to ensure that retinyl ester or carotenoid responses in the TRL fraction reflected absorption and metabolism of provitamin A carotenoids exclusively from the test meal, rather than from a previous meal. Two control trials of test meals containing 20 g fat but no carotene or vitamin A were performed and resulted in virtually no TRL response for either β-carotene or d_0-RE.

AUC values used to estimate the masses of RE and β-carotene derived from the test meals were calculated by using baseline-corrected concentrations. We made these corrections because baseline concentrations of RE and β-carotene in the TRL fraction were occasionally significantly above 0, which could not be ignored. Baseline concentrations of RE and β-carotene were 12 ± 7% and 16 ± 14%, respectively, of the observed peak TRL concentrations in the high-fat trials.
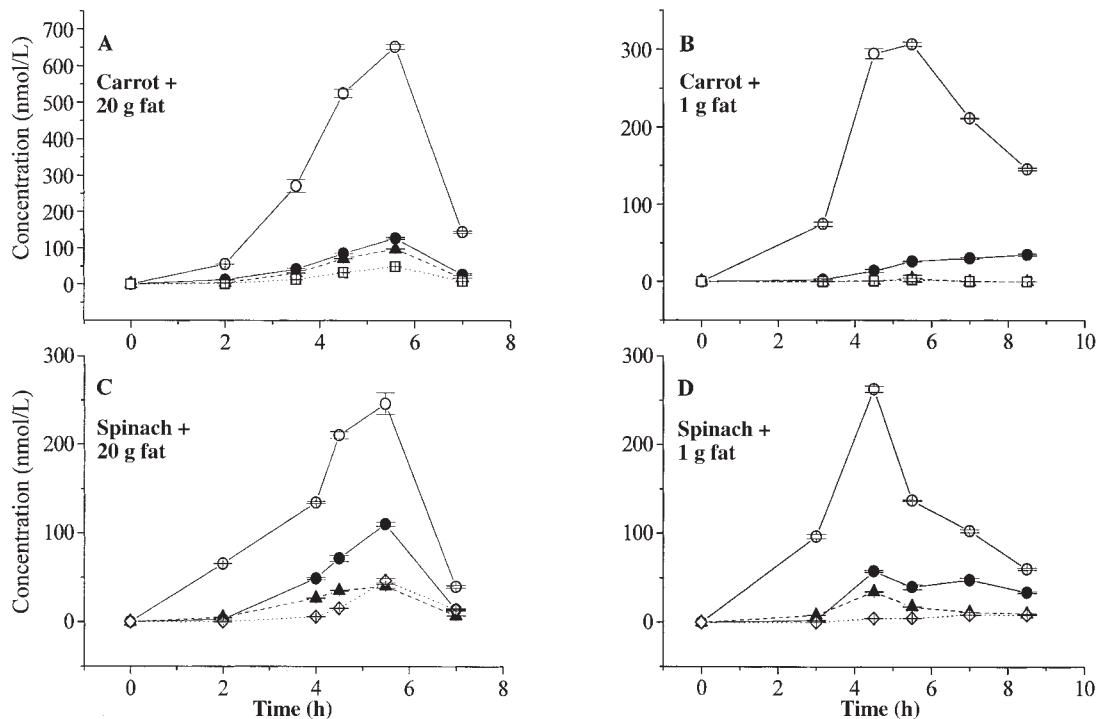
FIGURE 1. Mean ($\pm$SD) baseline-corrected concentrations of labeled (d_4; $\bigcirc$) and unlabeled (d_0; $\bullet$) retinyl esters and carotenoids [β-carotene ($\blacktriangle$), α-carotene ($\square$), and lutein ($\diamond$)] in the triacylglycerol-rich lipoprotein fraction of 2 subjects fed test meals containing raw carrot or raw spinach plus 6.0 μmol d_4-retinyl acetate in either 1 or 20 g safflower oil. A midday meal containing the same amount of fat as the test meal was provided 3.25 h later. Panels A and B show data from a subject fed 63 g raw carrot, with each amount of fat, in 2 separate trials (note that the y axis scale differs between panels A and B). The high-fat test meal (A) contained 9.78 μmol β-carotene, 6.39 μmol α-carotene, and 20 g fat; the low-fat test meal (B) contained 12.55 μmol β-carotene, 8.39 μmol α-carotene, and 1 g fat. Panels C and D show data from a different subject fed 122 g raw spinach, with each amount of fat, in 2 separate trials. The high-fat test meal (C) contained 9.82 μmol β-carotene, 7.68 μmol lutein, and 20 g safflower oil; the low-fat test meal (D) contained 11.83 μmol β-carotene, 6.76 μmol lutein, and 1 g safflower oil. Triplicate determinations were performed by analyzing 3 different plasma aliquots per time point.

Recovery of plasma RE in the TRL fraction ranged from 87% to 96% of the total RE present in 4 mL plasma. Additional analyses of unsaponified TRL fractions showed that amounts of retinol were undetectable.

Proportion of β-carotene dose absorbed from the test meals

Absorption of intact β-carotene from the high-fat vegetable test meals, expressed as a percentage of the 6 mg β-carotene ingested, ranged from 0.5 to 7.8% ($\overline{x}$: 2.9%). We also estimated the total amount of β-carotene assimilated from each test meal as a combination of intact β-carotene and d_0-RE. For the high-fat treatments, assuming central cleavage of β-carotene, the mean β-carotene uptake was $7 \pm 5\%$ of the dose (range: 3%–16%). If a stoichiometry of conversion of 1:1 was assumed, the calculated percentage of β-carotene taken up from the high-fat vegetable meals increased to $11 \pm 7\%$.

Influence of midday-meal fat content on TRL RE and β-carotene responses

The effect of the midday-meal fat content on the absorption of RE and β-carotene from a morning carrot test meal is shown for a single subject in **Figure 4**. The peak TRL concentrations and estimated masses of retinol and β-carotene absorbed were greatest when 20 g fat was present in both the morning and midday meals (high-fat, high-fat) and were lowest when 1 g fat was present in both meals (low-fat, low-fat). However, when a low-fat

morning test meal was followed by a high-fat midday meal, RE and β-carotene concentrations in the chylomicron fraction increased dramatically within 1.5 h of the midday meal and RE remained elevated at 8.5 h, when the trial was terminated. It is not known whether the masses of retinol and β-carotene derived from the low-fat, high-fat treatment would have approached the values from the high-fat, high-fat treatment if we had continued sample collection beyond 8.5 h.

DISCUSSION

Here we describe an improved method for estimating the mass of vitamin A absorbed after single meals containing provitamin A carotenoids. This approach combines features of the postprandial TRL-response model with the advantages of a labeled vitamin A reference standard. The approach is based on 4 elements. First, newly absorbed vitamin A and β-carotene are known to be assimilated exclusively via chylomicrons and occur in small chylomicron remnants or VLDL-size particles at lower fat intakes (15, 16). With the present method, 87–96% recovery of RE was obtained and there was virtually no RE or β-carotene response to test meals that did not contain β-carotene. Second, newly assimilated RE are not secreted from the liver in VLDL and do not readily exchange between chylomicrons and other lipoproteins; therefore, the baseline-corrected TRL RE response reflects only vitamin A derived from the test meal. Retinyl esters have been used for >20 y as a
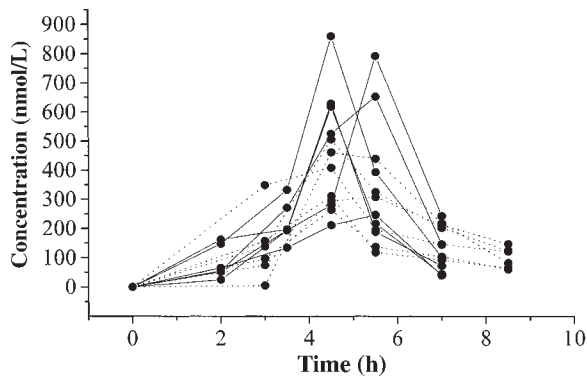
FIGURE 2. Postprandial concentrations of deuterium-labeled retinyl esters in the triacylglycerol-rich lipoprotein fraction of 3 subjects who each consumed 4 test meals containing 6.0 μmol d_4-retinyl acetate in an oil solution. Each line represents a different trial (*n* = 12); high-fat and low-fat test meals are indicated by solid and dashed lines, respectively.

marker for chylomicrons and their remnants (17–20). Third, labeled RE derived from the d_4-RA reference are transported in a manner similar to that of unlabeled RE derived from provitamin A carotenoids in the same meal. This was supported by the similar patterns of appearance and clearance of labeled and unlabeled RE (and β-carotene) observed under conditions of ample fat in the meal. Fourth, if an absorption efficiency is assigned to the d_4-RA reference dose, the TRL responses (AUCs) for labeled and unla-

beled RE can be used to estimate the mass of the latter that is derived from provitamin A carotenoids in the test meal.

We estimated absorbed vitamin A on the basis of the assumption that the d_4-RE AUC represented a consistent 80% of the 2-mg reference dose of d_4-RA. This seems reasonable in light of previous studies of retinol absorption in humans (21–23). Retinol is taken up by the intestinal mucosa by facilitated transport (24), which suggests that its absorption efficiency would be relatively refractory to meal conditions as observed by Borel et al (15) across a wide range of meal fat contents. The assumption of consistency of d_4-RA absorption was also invoked in the isotope-dilution method of estimating vitamin A stores (25–27) and of β-carotene conversion to vitamin A (28). In the current study, variation in the d_4-RE AUC was not related to the treatment and more likely reflected variation in in vivo chylomicron clearance kinetics.

The TRL-response model lacks a way of accounting for differences between trials in in vivo chylomicron kinetics. This is an important issue because the instantaneous plasma (TRL) concentration of newly absorbed retinyl esters or carotenoids is a function of not only the extent of uptake of these substances from the test meal but also their rate of clearance by the liver. The rate of chylomicron remnant clearance is known to vary substantially between individuals (17) and may also vary within the same individual on different days. For this reason, it is imperative that the labeled vitamin A reference dose be administered simultaneously with the test meal. In the present study, the d_4-RA reference also automatically controlled for variation in RE recovery during
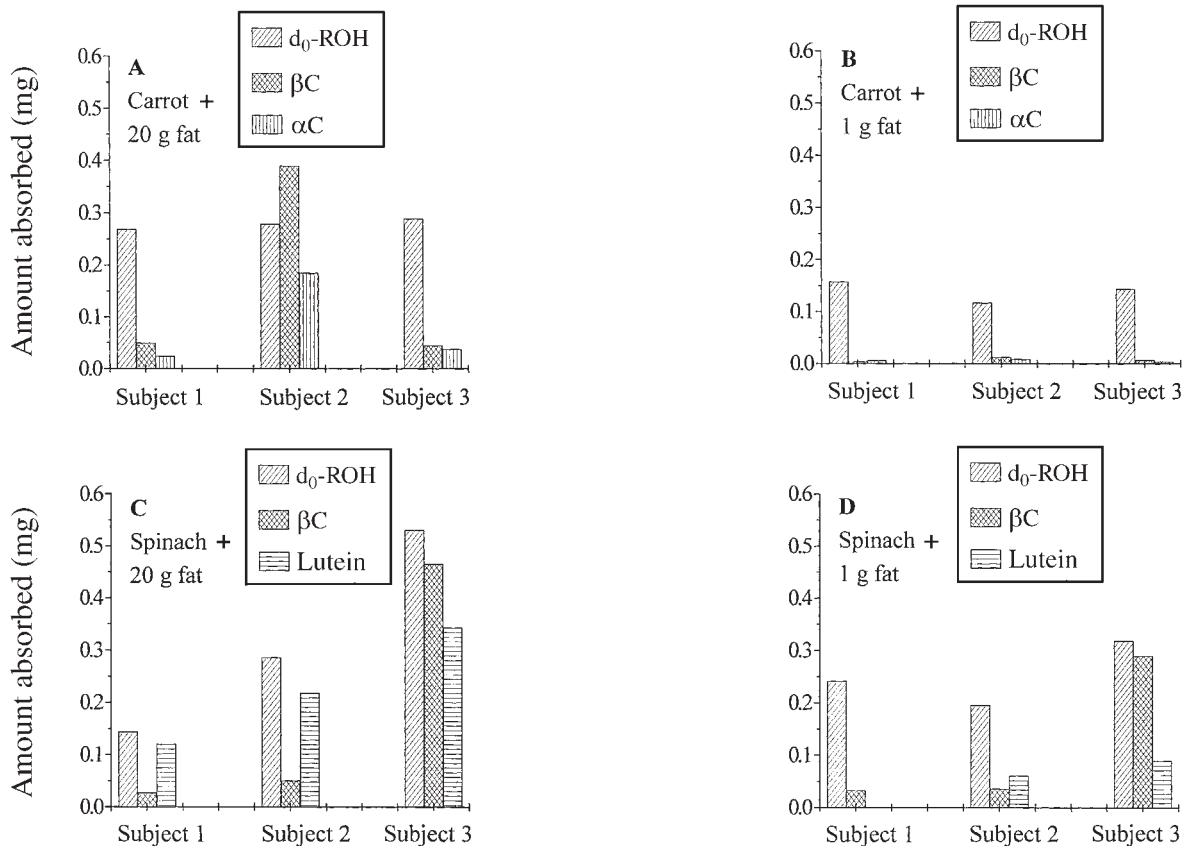

FIGURE 3. Estimated amounts of unlabeled retinol (d_0-ROH), β-carotene (βC), α-carotene (αC), and lutein absorbed after carrot and spinach test meals containing ≈6 mg *all-trans*-βC. The amounts absorbed were calculated by comparison with the d_4-retinyl acetate reference dose. Results were normalized to a 6.0-mg dose of βC for comparison purposes. Each of 3 subjects received 4 different treatments, in random order, 2–6 wk apart.
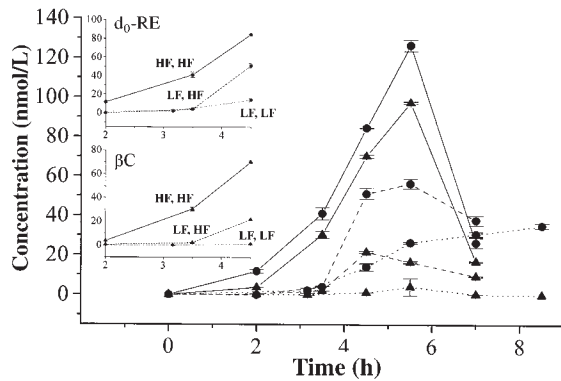
FIGURE 4. Mean (±SD) baseline-corrected concentrations of unlabeled retinyl esters (d_0-RE; ●) and β-carotene (βC; ▲) in the triacylglycerol-rich lipoprotein fraction of a subject who consumed carrot test meals on 3 separate occasions to show the effect of midday-meal fat content. HF, HF, high-fat (20 g) morning test meal followed by a high-fat midday meal (solid line); LF, LF, low-fat (1 g) test meal followed by a low-fat midday meal (dotted line); LF, HF, low-fat test meal followed by a high-fat midday meal (dashed line). Triplicate analyses were performed at each time point.

ultracentrifugation and subsequent sample-preparation steps, acting essentially as an assay internal standard. Whereas the non-d_4-normalized d_0-RE AUC values for a given treatment often varied considerably, the d_4-RE-normalized estimates of absorbed d_0-RE from these meals were more consistent.

Low-fat conditions resulted in muted and prolonged TRL-response patterns such that the entire absorption episode was apparently not captured within the 8.5-h time frame. Therefore, an extended postdose observation period may be required after low-fat test meals. Interestingly, the primary effect of low meal fat on absorption appeared to involve chylomicron secretion, rather than mucosal β-carotene uptake, because a high-fat midday meal stimulated RE and β-carotene absorption after a low-fat morning test meal. Although these healthy subjects assimilated substantial amounts of vitamin A and β-carotene under low-fat conditions, subjects who are less well nourished or are infected with parasites might be more adversely affected. Complete absence of fat severely reduces the plasma β-carotene response to single doses of β-carotene (29), whereas 3 g fat was as effective as 36 g fat in a 7-d β-carotene supplementation study (30).

In the high-fat trials, the estimated mass of retinol derived from the raw carrot or spinach carotenes ranged from 0.14 to 0.51 mg, considerably lower than the 1.0 mg predicted by the conventional 6:1 mass equivalency ratio of foodborne β-carotene to metabolically derived retinol (31). The mean observed equivalency ratio for these raw vegetables (ample fat condition) was 27:1, or 23:1 if the potential contribution of α-carotene was ignored. Intervention studies in vitamin A–deficient populations have led to proposed β-carotene:retinol mass equivalency ratios of 12:1 for fruit and 26:1 for cooked green leafy vegetables and carrots (32).

The estimated mass of β-carotene absorbed intact varied more widely than that of retinol, ranging from 0.03 to 0.47 mg in the high-fat carrot and spinach trials. In all but 1 of the 12 trials, the mass of absorbed retinol was greater than that of β-carotene. The mass ratio of retinol to β-carotene was not constant, either between or within subjects, ranging from 5.8:1 to 0.7:1 among the 6 high-fat trials. These differences may reflect

variation in intestinal β-carotene metabolism or in the extent of coflotation of hepatic VLDL-associated β-carotene in the TRL fraction. The latter would result in overestimation of the amount of β-carotene absorbed intact. However, VLDL- β-carotene was reported to peak 7–9 h after an oral dose of β-carotene (8, 33, 34), at which time the TRL β-carotene concentrations in the present study had nearly returned to baseline. Additionally, the ratios of RE to β-carotene observed in this study were similar to those measured by van Vliet et al (4), who used ultracentrifugation conditions expected to minimize contamination of TRL by hepatic VLDL.

Assuming 2 mol RE/mol β-carotene (35), our results indicated that a mean of 7% (range: 3%–16%) of ingested β-carotene from these raw vegetables, consumed with ample fat, was taken up as the sum of RE and β-carotene. Although these values are lower than that predicted by the conventional assumption of 33% absorption of β-carotene from food, they are not unexpected. Using different methodologic approaches, van Vliet et al (4), Novotny et al (36), and Lin et al (28) estimated β-carotene uptake values of 11%, 22%, and 6%, respectively, from β-carotene supplements. Earlier lymph recovery studies in humans yielded absorption efficiencies of 9–17% (23, 37). Although oral-fecal balance models occasionally indicated higher β-carotene absorption efficiencies, up to nearly 100% (3), the inability to control for chemical or microbial degradation of carotenes in the gut complicates their interpretation.

The conventional β-carotene-to-retinol mass equivalency ratio of 6:1 involves an assumption of 50% conversion efficiency and a prediction that equivalent masses of β-carotene and retinol will be absorbed. In 9 of our 12 trials, subjects absorbed substantially more retinol (as RE) than unmetabolized β-carotene, consistent with observations of van Vliet et al (4). This suggests that conversion efficiency commonly exceeds 50%, even in well-nourished individuals. Thus, the primary factor responsible for the lower-than-predicted vitamin A values of raw carrot and spinach was probably poor intestinal uptake, rather than inefficient intestinal conversion of β-carotene to retinol.

Currently, there is no gold standard with which to evaluate a technique for determining the vitamin A potential of specific foods. Here we describe a novel method to empirically estimate the mass of vitamin A derived from a single meal. The method, which uses conventional bench top gas chromatography–mass spectroscopy, provides a simultaneous comparison of the vitamin A values of preformed vitamin A and foodborne provitamin A carotenoids. Although it is not a substitute for field-based efficacy studies, this method can be used to screen the vitamin A potential of different foods or supplements and identify processing techniques that have a major impact on the vitamin A value of these foods. The results to date support the notion that the average vitamin A value of at least some plant foods may be overestimated by the conventional β-carotene equivalency. Our findings indicate a need for more systematic evaluation of common foods, meal conditions, and cooking practices. ❧

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