A sensitive procedure for the study of β-carotene-d8 metabolism in humans using high performance liquid chromatography–mass spectrometry

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Abstract **This report describes the development of a robust method of high sensitivity for studying the metabolism of β-carotene-d8 in humans using a combination of liquid chromatography/particle beam–mass spectrometry (LC/PB-MS). The utility of the LC/PB-MS method was demonstrated in a pilot study. The carotenoids were extracted from plasma into hexane and separated by reverse phase high performance liquid chromatography (HPLC) using a C-18 column. The HPLC effluent was nebulized using helium and the solvent was removed under vacuum within the dual-stage particle beam interface. The de-solvated carotenoids were ionized in the negative-ion mode (electron capture) using methane chemical ionization and detected using selected ion monitoring. The limit of detection of the method was on the order of 0.3 ng (approximately 0.6 pmol) for β-carotene. β-Carotene-d8 was quantified in the plasma over a concentration range of two orders of magnitude using β-carotene-$^{13}C_{40}$ as an internal standard. The overall coefficient of variance (CV) for determining the concentration of the analytes from 30 μl of plasma was 3.9% for β-carotene and 2.4% for β-carotene-d8. Using the LC/PB-MS method, the concentration of β-carotene-d8 was determined in the plasma of a subject who had consumed a single 5-mg dose over a 30-day period. The sensitive semi-automated procedure is capable of high sample throughput and makes large comprehensive studies feasible.**—Pawlosky, R. J., V. P. Flanagan, and J. A. Novotny. **A sensitive procedure for the study of β-carotene-d8 metabolism in humans using high performance liquid chromatography–mass spectrometry.** ***J. Lipid Res.*** **2000.** 41: **1027–1031.**

Supplementary key words β-carotene • mass spectrometry • stable isotope • high performance liquid chromatography • metabolism

Current nutritional recommendations suggest that Americans should include five servings of fruits and vegetables daily in their diet. Many of these foods contain carotenoids, such as β-carotene, which may be an important source for vitamin A. There is a paucity of information on the metabolism of β-carotene in humans and current interest in this area is due, in part, to the pro-vitamin A properties of this compound.

Several reports have documented the development of analytical methods for studying the metabolism of labeled β-carotene in humans (1–3). Unfortunately, most of the reported methods involve laborious purification procedures. In one report, Dueker et al. (2) described a multiple-step procedure for determining the concentration of β-carotene-d8 in human plasma. After extraction into an organic solvent, carotenoids were resolved by high performance liquid chromatography (HPLC) on a reverse phase column. Individual fractions were collected, further purified, and then analyzed by tandem-mass spectrometry (using direct-insertion probe introduction into the mass spectrometer). Another analytical method for β-carotene-$^{13}C_{40}$ reported by Parker et al. (3) describes a multiple-step reverse phase HPLC procedure. The individual sample fractions, which eluted within the retention time corresponding to β-carotene had to be collected, then underwent hydrogenation prior to analysis by gas chromatography–combustion–isotope ratio mass spectrometry (GC-C-IRMS). Both of these approaches involve lengthy procedures consisting of extraction, purification, and/or sample hydrogenation prior to mass spectral analysis. Unfortunately, neither approach is an in-line method insofar that HPLC fractions of the analytes must first be collected prior to analysis by mass spectrometry.

A comprehensive study design to determine the metabolic fate of β-carotene in humans requires an experimental procedure of high sensitivity as well as high sample throughput. We describe here the development of a robust and quantitative method for studying the metabolism of β-carotene-d8 in humans using HPLC with in-line particle beam–negative ion–mass spectrometry (PB-NCI-MS)

Abbreviations: LC/PB-MS, liquid chromatography/particle beam–mass spectrometry; GC-C-IRMS, gas chromatography–combustion–isotope ratio mass spectrometry; PB-NCI-MS, particle beam–negative chemical ionization–mass spectrometry

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for detection. The utility of the LC/PB-MS method was demonstrated in a pilot study in one subject who had consumed a 5-mg dose of the isotope.

MATERIAL AND METHODS

Materials

All *trans*-β-carotene-10, 10′, 19, 19, 19, 19′, 19′, 19′ -d8 (β-carotene-d8) having a chemical of purity of greater than 87% as determined by LC-MS and a stated isotopic purity of approximately 98% was purchased from Cambridge Isotope Labs (Woburn, MA). All *trans*-β-carotene-U $^{13}C_{40}$ with an isotopic purity of 98% or greater and chemical purity of approximately 85% was purchased from Martek Co. (Columbia, MD). The major chemical impurities in the β-carotene-U $^{13}C_{40}$ were the 9 and 13 *cis*-isomers, which were partially resolved from the all *trans* isomer (see fig. 2). A multiplication factor of 0.85 was used when quantifying β-carotene-d8 in the plasma. All solvents that were used in the study were HPLC grade and were used without further purification.

Human study design

The human study was approved by the Institute's Review Board of the Beltsville Human Nutrition Research Center. One subject was given a 5-mg oral dose of β-carotene-d8 that was mixed into a breakfast beverage drink prior to the morning meal. Blood was drawn from the forearm of the subject at 0 h, 8 h, 8 d, 15 d, 21 d, and 29 d and the plasma was separated from the blood cells using a clinical centrifuge. Without further fractionation, the plasma samples were then frozen in 1-mL aliquots and stored at −80°C until they were extracted and analyzed.

Plasma sample preparation and extraction procedure

Plasma specimens (1 mL) were thawed and 372 ng of the internal standard (ISTD), β-carotene-$^{13}C_{40}$ (in 9 μL of CH_2Cl_2) was then added into each sample. After the addition of the internal standard, samples were equilibrated for 30 min at 0°C. Seven hundred fifty μL of the plasma was added to 750 μL of a BHT–ethanol solution (butylated hydroxyl toluene; 50 mg/L) and 1.5 mL of hexane in 5-mL glass tubes. The tubes were vortexed for 30 sec and then centrifuged at 2,000 rpm for 5 min in a table-top centrifuge. The hexane layer was filtered through a 25-mm syringe filter-0.45 micron PVDF (Alltech Associates Inc., Deerfield, IL) into a clean test tube and the aqueous layer was extracted twice with an additional 1.5 mL of hexane. The hexane layers were combined and evaporated to dryness under a stream of nitrogen. The samples were dissolved in 500 μL of the HPLC solvent mobile phase and 20 μL was taken for injection. The remaining aliquots were stored at −60°C.

HPLC-MS experimental conditions

A 20-μL aliquot of the hexane-extracted plasma (equivalent to 30 μL of plasma) was injected onto a 250 × 0.46 mm 5 micron C-18 Microsorb column (Rainin Instrument Co., Woburn, MA) that was fitted to a Beckman model-110 HPLC system. The mobile phase consisted of CH_3CN:CH_2CL_2:CH_3OH having a

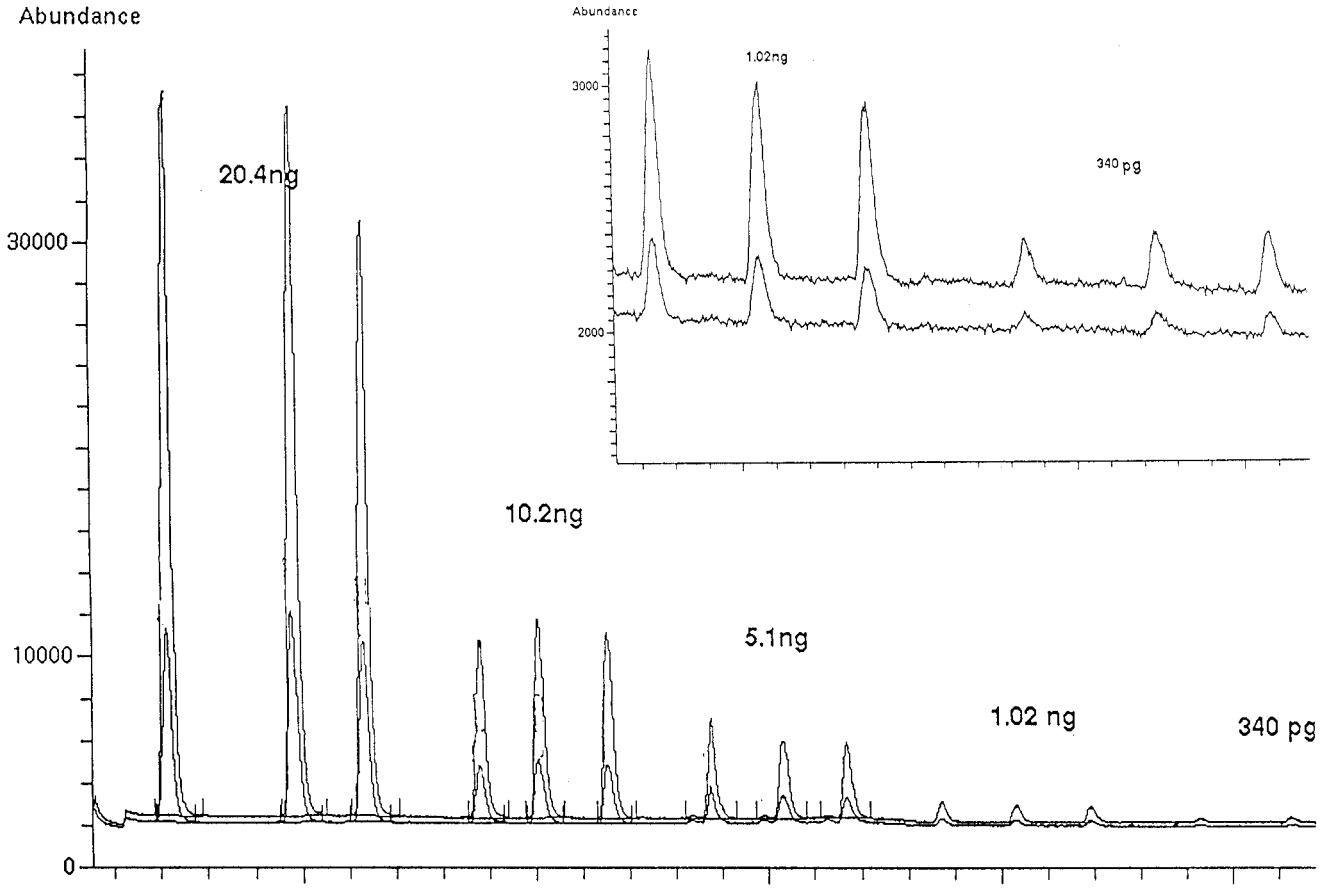

Fig. 1. Selected ion chromatograph (*m/z* 544) for the repeated injections (20 μl) of β-carotene-d8 by particle beam LC-MS using negative ion conditions with methane. The amounts of the pure standard injected ranged from 20.4 ng to 340 pg of the analyte.

ratio of 65:25:10 (+ 0.1% diisopropylethylamine). With a flow rate of 0.5 mL/min, the retention time for all *trans* β-carotene was 30.2 min. The effluent was delivered directly into the particle beam interface (model 59980A) of a Hewlett-Packard 5989B mass spectrometer and the solvent stream was nebulized with helium (with a pressure of 60 psi) directed orthogonal to the incoming solvent stream. The solvent was removed under vacuum within the two-stage particle beam separator and the de-solvated carotenoids were ionized using negative chemical ionization (NCI) source with methane (ion source pressure and temperature 1.5×10^{-4} torr and 250°C.). The data acquisition program (Hewlett-Packard UNIX Chem Station) was set to scan in the selected ion mode for ions at *m/z* 536 (the molecular ion of unlabeled β-carotene), 544 (β-carotene-d8), and 576 (β-carotene-$^{13}C_{40}$) using a dwell time of a 1000 millisec for each of the ions.

Reproducibility, limits of detection, and precision of the method

Reproducibility and limits of detection for the LC/PB-MS method were determined from values obtained from repeated injections of the pure standard. Several repeated injections of β-carotene-d8 were made ranging from 20.4 n to 340 pg (see Fig. 1). The precision of the method for β-carotene-d8 that was spiked into pooled plasma (plasma obtained from several subjects) at a concentration of 100 ng/mL and β-carotene was determined from repeated injections of four separate plasma extractions. The means and coefficients of variation for the ratios of β-carotene-d8/ISTD and β-carotene/ISTD were calculated from the chromatographic peak area counts of each analyte.

Accuracy determinations-standard dilution curve for β-carotene-d8 in plasma

A standard dilution curve for quantifying β-carotene-d8 in plasma was prepared using β-carotene-$^{13}C_{40}$ as the internal standard to quantify the d8-isotope. To each of six plasma samples (1 mL each) a serial dilution of β-carotene-d8 was added. In the plasma the concentration of β-carotene-d8 ranged from 3.0 to 300 ng/mL. β-Carotene-$^{13}C_{40}$ (372 ng) was added to each plasma sample and all the samples were then extracted and analyzed by LC/PB-MS. The ratios obtained from the chromatographic peak area counts for β-carotene-d8/β-carotene-$^{13}C_{40}$ were plotted against the known concentration of β-carotene-d8 in the plasma sample.

RESULTS

The LC/PB-MS method was developed for the purpose of making large-scale stable isotope studies in humans feasible. A review of the various β-carotene mass spectrometry methods (1–5) points out several drawbacks in the current paradigms, such as lack of sensitivity (4) or complicated experimental design (1–3). The particle beam method is sensitive, having a detection limit of about 300 pg for β-carotene (**Fig. 1**). This detection limit, which compares favorably to that obtained using electrospray mass spectrometry (4), was similar to those LC/PB-MS values reported by Careri and Manini (6). This high

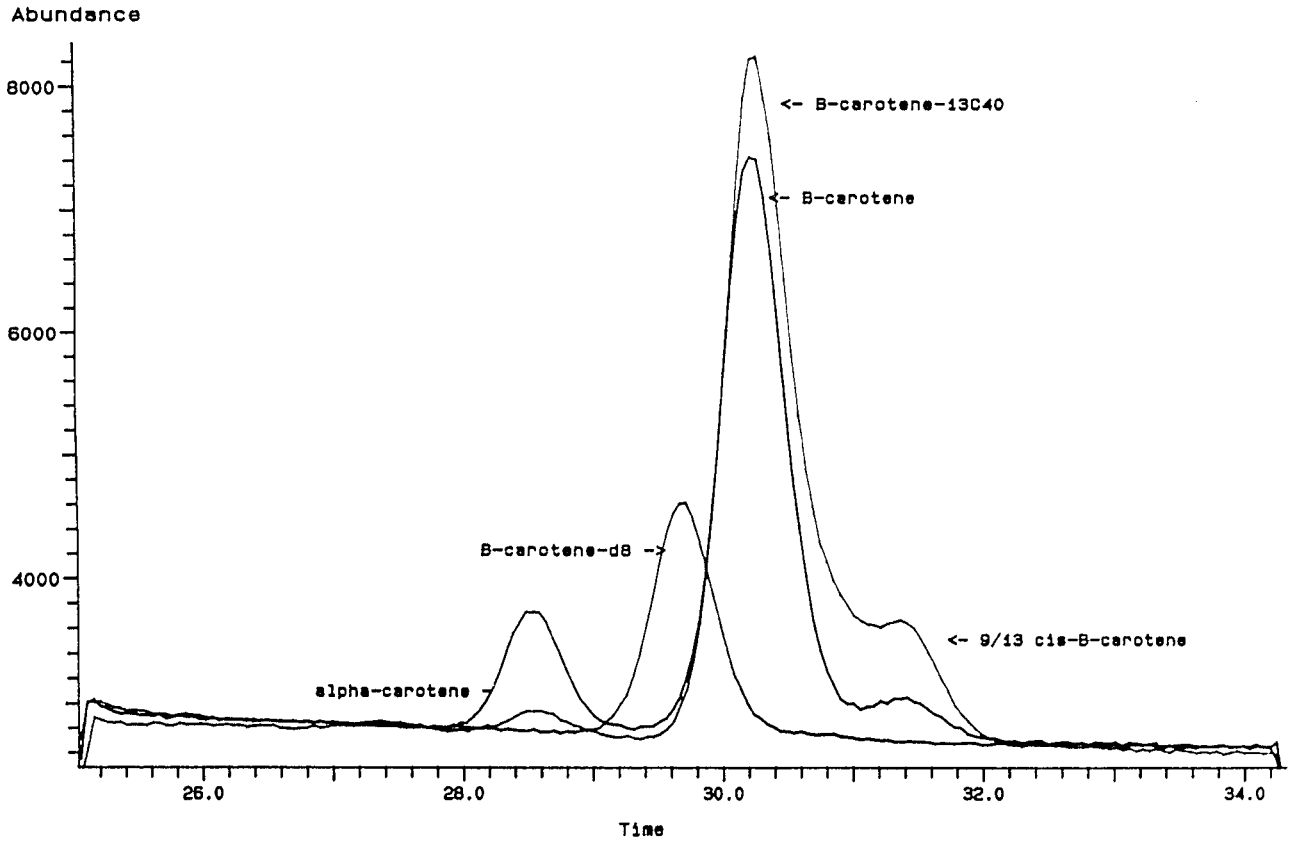

Fig. 2. Selected chromatograph tracings for β-carotene, β-carotene-d8, and β-carotene-$^{13}C_{40}$ by LC-MS from the 8-h time point plasma sample of a subject who had consumed 5 mg of β-carotene-d8. β-Carotene-d8 elutes 29.8 min, just prior to β-carotene and β-carotene-$^{13}C_{40}$. The signal represents the equivalent of 30 μL of plasma injection onto the LC.

degree of sensitivity associated with negative chemical ionization may result from a combination of factors, such as the resonance properties of the conjugated polyene system as well as the moderating effects of the reagent gas, which contribute to stabilizing the radical anion (7). In addition, the low amount of fragmentation of the molecular ion tends to concentrate virtually the entire ion current into a single ion (6). Whereas, in using electrospray ionization (positive ion detection), formation of the molecular ion of the carotenoid is influenced by the alkaline properties of the analyte (Bronstead-Lewis base) relative to the acidic strength of the solvent. The formation of the M^+ ion of β-carotene requires the addition of a mobile phase adjuvant to oxidize the carotenoid (4). The adjuvant, however, may contribute to the formation of multiply charged ions, which may complicate the analysis and decrease sensitivity.

The particle beam interface is of rugged design, a factor that contributed to the reliability of the mass spectrometry method. Essentially, the only adjustment that was required was to the length of the capillary tube that extended through the stainless steel guide into the interface (about 1 mm beyond the guide for optimal sensitivity). Other details pertaining to the operation of the HPLC/PB-MS system are described in the above section.

The reproducibility of the LC-MS method as determined from repeated injections of differing concentrations of β-carotene-d8 is given in Fig. 1. The limit of detection for the method based on a signal-to-noise ratio of 3:1 was about 300 pg of the pure standard. The precision of the method for β-carotene-d8 and β-carotene from plasma analysis was determined to be 2.4% and 3.9%, respectively, based on four repeated injections of samples.

Serial dilutions of β-carotene-d8 in plasma were plotted as the ratio of the area counts of β-carotene-d8/ISTD throughout a concentration range which varied from 3 to 300 ng of analyte per mL of plasma. The graph of the dilution curve conformed to a linear regression using a least squares fit ($y = 0.763x + 2.32$) with an R^2 value of 0.999. This demonstrated that the method could be reliably applied to the quantification of β-carotene-d8 throughout this range.

The plasma concentration of β-carotene-d8 from a subject who had consumed a single 5-mg dose of the isotope was determined over a 30-day period. The plot of the ion current tracings over the analysis time for β-carotene, β-carotene-d8, and β-carotene-$^{13}C_{40}$ are presented in **Fig. 2**. The graph demonstrating the time-course determinations for β-carotene-d8 is given in **Fig. 3**. At the zero time point there was no detectable interference in the ion channel (*m/z* 544) corresponding to β-carotene-d8. It appeared that the concentration of β-carotene-d8 reached a maximum concentration in the plasma at the 8-h time point. To determine the absolute maximum concentration of β-carotene-d8 in the plasma, several more blood samples at various time points within the first 24 h would be needed. The results from the pilot study demonstrate the effectiveness of the LC/PB-MS technique in accurately determining minute concentrations of the isotope in plasma.

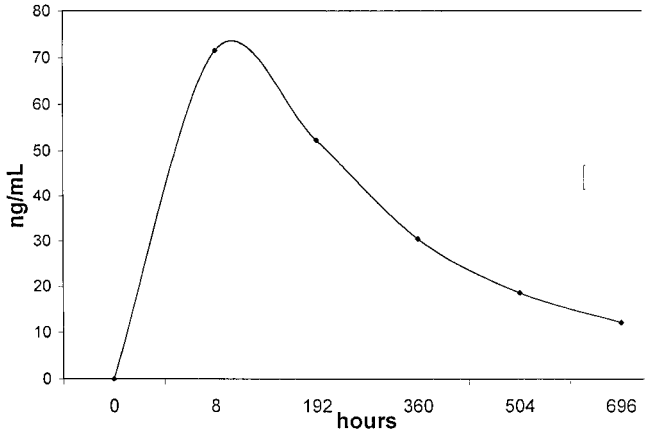

Fig. 3. The time-course for plasma concentration of β-carotene-d8 from one subject who had consumed a 5-mg dose of the labeled compound. Samples were analyzed by particle beam-LC-MS and quantified using β-carotene-$^{13}C_{40}$.

DISCUSSION

The development of an uncomplicated and sensitive method to study the metabolism of β-carotene in humans is of high priority. The existing mass spectrometry techniques that have been exploited for this purpose are time-consuming and require multiple sample manipulation steps (1–4). The need for an efficient, sensitive, and robust method to be used in human studies has been met with the development of the LC/PB-MS method. The method has several important advantages over the current techniques. A single extraction step into hexane was sufficient to isolate the carotenoids. It was not necessary to collect HPLC fractions of the carotenoids for further purification. The LC effluent was delivered directly to the mass spectrometer for analysis through the particle beam interface. The ionization process exploited the chemical structure of the carotenoid to produce a high signal-to-noise ratio. Sample hydrogenation (3) or the use of mobile phase adjuvants (4) were not necessary. The current method advances a straightforward experimental design for the analysis of β-carotene and β-carotene-d8. The utility of this method has been demonstrated in a pilot study involving one subject who consumed a small dose of β-carotene-d8. The advantages of this new technique are now being exploited in a comprehensive study examining the fate of β-carotene-d8 in humans.

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