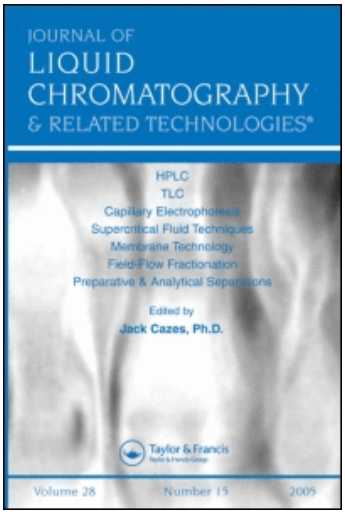

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Individual Carotenoid Determinations in Human Plasma by High-Performance Liquid Chromatography

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INDIVIDUAL CAROTENOID DETERMINATIONS IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A high-performance liquid chromatographic technique is described to quantify beta carotene from alpha carotene and lycopene in human plasma. Total analysis time is 14.5 min. A reverse-phase column was employed with a mobile phase composed of 65% acetonitrile: tetrahydrofuran (90:10, v:v) in methanol. Use of the internal standard, beta-apo-8- carotenoic ethyl ester permitted a reliable way to quantify potential losses in plasma extractions. Plasma beta carotene levels obtained from subjects several days after supplement use were observed to increase three-fold or more.

INTRODUCTION

Determinations of serum beta carotene in clinical laboratories have relied largely upon the measurement of total carotenoids via absorbance at a fixed wavelength (1-3). This nonspecific technique was employed in a recent study which examined serum carotenoids from patients who were diagnosed as having cancer in the subsequent five years and similar patients who remained cancer-free. Consequently, one could not ascertain which if any of the individual carotenoid level(s)

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were different in cases versus controls; no significant difference between these two groups were found with total carotenoid measurements. As Peto and others (5,6) have recommended, the epidemiologic evidence for the lung cancer-beta carotene hypothesis can only be tested if clinical studies incorporate more discriminatory analyses of carotenoids in foods and tissues. Secondly, research must establish whether a single blood determination (or dietary estimate) is truly reflective of the usual dietary intake patterns over the period of carcinogenesis.

The predominant carotenoids found in human plasma or sera include alpha carotene, beta carotene and lycopene along with possibly an oxy-carotenoid or xanthophyll named cryptoxanthin (7,8). Therefore, analyses of fluids should incorporate determinations of these compounds. Several earlier reports in the literature (9-11) have examined the correspondence between prescribed test meals or beta carotene supplement use and plasma/serum total carotenoids concentrations in a number of human subjects. A preliminary investigation was conducted in this laboratory to evaluate the relationship between short term supplement use and plasma levels with a method that quantifies beta carotene levels apart from those of other carotenoids.

Previously reported separations of carotenoids were primarily in food matrices by long, laborious open-column chromatographic techniques (12-14) or with rather complicated hardware setups for greater speed of analysis and normal phase high-performance liquid chromatography (15,16). With the advent of bonded phase columns, specifically, the so-called reverse phase columns, the analyses of complex mixtures of carotenoids and/or xanthophylls became possible (8, 11-19). Alpha and beta carotenes as geometric isomers are probably the most difficult pair of compounds to separate when considering human tissue specimens. The method described herein was developed for the quantitative analyses of these compounds in

such matrices. The method development was also intended to quantify other vitamins potentially protective against human cancer, in particular, the fat-soluble vitamins A and E. The following attributes have led to routine use of the method in our laboratory: 1) the resolution of beta and alpha carotenes; 2) the use of an internal standard structurally similar to the carotenoids of interest and; 3) a standard protocol to mitigate storage losses. To the best of our knowledge, only two reports in the literature use an internal standard (21,22) and neither include a scheme to separate alpha carotene from beta carotene.

MATERIALS AND METHODS

Materials

L-ascorbic acid, beta hydroxytoluene (BHT), all-trans beta carotene, all-trans alpha carotene, lycopene, retinyl acetate, and all-trans retinol were obtained from Sigma (St. Louis, MO). Alpha and beta carotene were of the highest purity available, types V and IV, respectively. Azobenzene and retinyl palmitate were obtained from Fluka (Hauppauge, NY). Alpha and gamma tocopherols (both d-), dl-alpha tocopheryl acetate, dl-tocol, canthaxanthin and beta apo-8-carotenoic acid ethyl ester (BcEE) were generous gifts from Dr. W.E. Scott, Hoffman LaRoche Inc. (Nutley, NJ). All standards dry or as solutions were stored in amber bottles under N_2 at - 20°C. Crystals of alpha and beta carotene, retinyl palmitate, and lycopene were first dissolved in hexane/acetone (60/40, w/v) solutions. Subsequent dilutions for working calibration mixtures were made with the mobile phase. For the tocopherols, initial dilutions were made with absolute ethanol, then with methanol. Retinol and retinyl acetate were dissolved in methanol directly. All solvents were HPLC-grade (Omnisolve, MCB Manufacturing Chemists, Inc., Gibbstown, NJ) and degassed by vacuum filtration prior to use.

Liquid Chromatography

The liquid chromatographic system included two reciprocating pumps (Model 510) and an automatic gradient controller (Model 680, Waters Associates, Milford, MA); a Rheodyne 7125 sample valve injector with a 50ul loop (Cotati, CA); a Perkin-Elmer LC-75 variable UV-visible spectrophotometer (Norwalk, CT) set at 292nm or 450nm and 0.04 aufs and; a Hewlett Packard 3390A reporting integrator (Avondale, PA). Two types of stainless steel columns were operated at ambient temperatures - a 5um Ultrasphere ODS column (Altex Scientific, Berkeley, CA), 250mm long x 4.6mm id and a 3um Supelcosil LC-18 (Supelco, Inc., Bellefonte, PA), 150mm long x 2.9mm, id. A Brownlee guard column holder with a 10um ODS cartridge (Santa Clara, CA) was placed in series between an inline solvent filter and the analytical column; cartridges were replaced monthly to obviate contamination of the analytical column.

Initial experiments were performed to achieve capacity ratios (K') between 2 and 10. K' was defined as t_R-t_o/t_o, where t_R and t_o were the retention times of the compound of interest and the unretained peak, respectively. Various strategies were tested in order to optimize the resolution (Rs) between alpha and beta carotene and/or alpha and gamma tocopherols. Rs was defined as follows:

$$Rs = (t_{R2} - t_{R1})/(w_2 + w_1)$$

where t_{R2} and w_2 represented the retention time and peak width of beta carotene (or alpha tocopherol), respectively, and t_{R1} and w_1, the corresponding parameters for alpha carotene (or gamma tocopherol).

The gradient program on the Ultrasphere column was as follows: pump 5% water in methanol (100% A solvent reservoir) for the first 14 min, change linearly to 4% tetrahydrofuran in methanol (100% B) over 2 min and maintain for another 14 min and return to 100% A over the next 3 min and maintain this

solvent composition for a minimum of 15 to 18 min for reequilibration. Eluents were monitored at 292nm for the first 22 min until the retinyl palmitate peak eluted; then the wavelength was quickly changed to 450nm for the carotenes. The total analysis time was 45 min. Isocratic analyses of retinol, retinyl acetate, and the tocopherols were also accomplished on the Supelcosil C-18 column within 14 min using 100% methanol mobile phase system described previously (23). Then isocratic analyses for carotenoids were conducted on the same Supelcosil column with 35% methanol in acetonitrile/tetrahydrofuran, (90/10, v/v); flow rates approximated 2ml/min.

Quantification and Calibration

A standard curve of peak height ratios (compound of interest to internal standard, BcEE or tocol) versus concentration ratios was calculated daily for every set of analyses. Calibration mixtures contained differing amounts of beta carotene, alpha carotene, and lycopene with a constant amount of the internal standard. Weight ratios were selected to reflect expected concentrations in samples studied. Linearity of calibration graphs was tested by analysis of variance using R^2 as the criterion of adequacy.

Extractions from Serum or Plasma, Recovery Efficiencies, and Storage Protocol

Serum or plasma samples (0.5ml) were placed in glass test tubes containing 0.1ml BcEE (10.56ug/ml), and 0.1ml 3% L-ascorbic acid in absolute ethanol. Three ml of 0.0125% BHT in hexane were added and contents were gassed under N_2, capped, mixed on a vortex mixer for 20s, and centrifuged to separate phases. The organic phase was removed and transferred to a new tube; the remaining aqueous infranatant was reextracted with 3ml of 0.005% BHT in hexane. The second organic layers were

pooled with the first and evaporated until almost dry under N_2. The residue was carefully rinsed with additional hexane, transferred to a microcentrifuge tube and evaporated to dryness with N_2. This concentrate was then quickly redissolved in 0.2ml mobile phase. All extractions were performed either under subdued light or red lights.

Dogs fed commercial lab chow were used for recovery experiments because no carotenoids were detected in their plasma. Recovery efficiencies were assessed by adding 1.225ug and 2.45ug beta carotene to dog plasma as well as plasma and sera from one human volunteer with detectable alpha and beta carotene. Six replicates were made at each level: beta carotene was added to three and not added to the other three.

Two specimens of fasting blood from one human volunteer, 20ml each were collected in EDTA-coated and anticoagulant-free vacutainer tubes. Plasma and sera aliquots were extracted as usual and analyzed by HPLC the same day (day 0). Remaining plasma and sera were divided into amber glass vials each containing approximately 1.5ml sample, gassed quickly with N_2 and frozen at -20°C for later analyses. Samples were extracted and analyzed again at 7, 18 and 48 days after venipuncture. No consistent differences were observed for beta carotene measurements analyzed at any time or between sera and plasma extracts. Broich et al (19) have reported similar observations. Stability of frozen sera and plasma with the additions of various antioxidants for longer time periods is currently being studied.

Description of Subjects and Supplementation Design

In attempt to determine what effect large doses of beta carotene have on plasma beta carotene levels of human volunteers, a time series-type design was established with four subjects. Fasting blood specimens were drawn on day zero just

prior to ingestion of one capsule containing 30mg Solatene® (Hoffmann-LaRoche, Inc. Nutley, NJ). This preparation was selected for its notably high bioavailability. No attempt was made to record or modify the subjects' dietary intakes. Subjects received either no, one, two or three capsules each morning from the investigator for a variable number of days. At several time points, subjects fasted 10-12 hr prior to the morning venipuncture. All samples were either extracted within 4 hr of venipuncture and analyzed the same day or gassed under a N_2 stream and frozen at -20°C until extraction and HPLC analyses could be performed (usually with 7 days).

RESULTS AND DISCUSSION

Chromatography

In order to simultaneously resolve compounds of similar as well as widely divergent lipophilicities, a gradient elution program was necessary. Figure 1 illustrates the separation a standard solution mixture of retinol, tocopherols, retinyl palmitate and the carotenes-alpha and beta. The Ultrasphere ODS column adequately resolved gamma and alpha tocopherols with a resolution (Rs) = 1.27, but the Rs between alpha and beta carotenes was somewhat poorer, Rs = 1.01. Note the proximity of the retinol peak to the solvent front (t_o). A small percentage of water (5%) was added to methanol (A reservoir) in order to retain the compound, K'>2. Greater retention was achieved by increasing the proportion of water, but the analysis time also increased. In addition, more water exacerbated the solubility limits for the carotene, leading to poorly shaped peaks and eventual pressure increments. The manual change in wavelength during the run and after the elution of carotenes also imposed several limitations. First the operator had to be present for the analyses. Second,

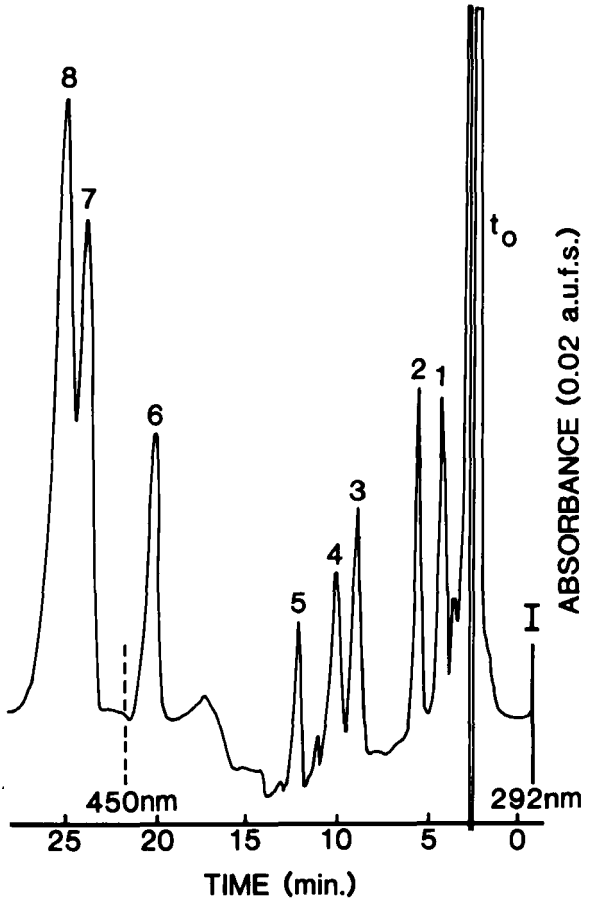

FIGURE 1: Representative HPLC profile of a standard mixture on the Ultrasphere ODS column. Conditions are described in text. t_o = solvent front; 1 = retinol; 2 = retinyl acetate; 3 = gamma-tocopherol; 4 = alpha-tocopherol; 5 = alpha-tocopheryl acetate; 6 = retinyl palmitate; 7 = alpha-carotene; 8 = beta-carotene.

because the selected carotenoid-like internal standards were more polar than the carotenes, they eluted when the detector was set at 292nm and thus were not optimally detected. For this reason, dl-alpha-tocopheryl acetate was used as the internal standard for all peaks of interest. Third, the

analysis time was quite long. In the investigator's opinion, the best solution for these drawbacks is to use a dual wavelength detector which was not available in our laboratory. With such a detector, no baseline correction would be necessary. Most importantly, with a single chromatographic injection and sample preparation, quantification of at least 8 different compounds would be possible. Further, the most chemically similar and appropriate internal standards---retinyl acetate for retinol, dl-tocol for the vitamin E compounds, and BcEE for the carotenoids could be used without interference or overlapping peaks. These internal standards are ideal because such compounds would not be present in analyzed samples.

Because of these equipment limitations in our laboratory, the second chromatographic method developed for the determination of carotenoids in tissues is illustrated by the chromatograms in Figures 2 and 3. Figure 2 depicts a chromatogram of a standard solution mixture. At a flow rate of 2.0 ml/min, the total assay time was 14.5 min. Two conditions primarily facilitated the faster elution times - a shorter column and a mobile phase of higher solvent strength (nonaqueous solvents only). The latter also permitted better sample solubility and lower operating pressures. However, with such fast elution profiles, the internal standards first attempted (azobenzene and canthaxanthin) were not sufficiently retained. BcEE with a K' = 2.11 was selected. The Rs for alpha and beta carotene was consistently between 1.88 and 1.90 at flow rates of 2.0 - 2.5ml/min. Figure 3A depicts a chromatogram of a plasma extract in which BcEE was added. This extract was also prepared with no internal standard added; no other compounds were observed to co-elute with this compound (Figure 3B). Figure 4 demonstrates the similarity in absorbance patterns between these carotenoids. Because these standards were dissolved in the mobile phase, a slight shift in

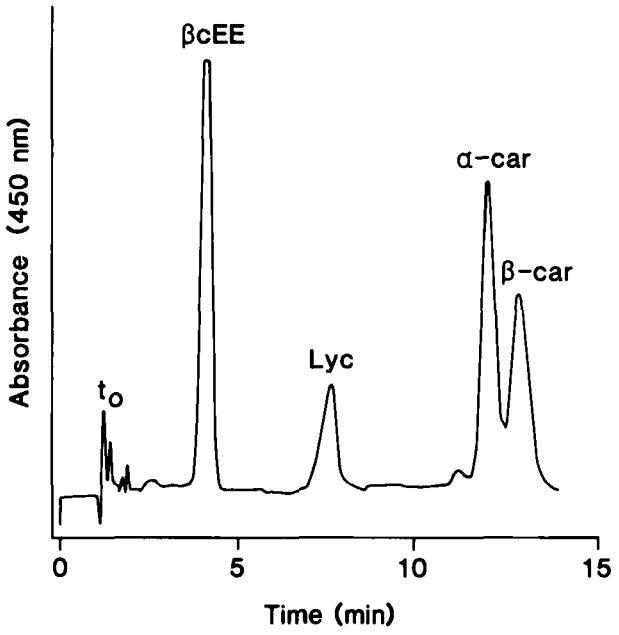

FIGURE 2: Representative HPLC profile of standard mixture on Supelcosil C-18 column. Conditions are described in text t_o = solvent front; BcEE = beta apo-8-carotenoic ethyl ester; Lyc = lycopene; α-car = alpha-carotene; β-car = beta-carotene.

wavelength maxima was observed in comparison to reported values in other solvents (17,24). Several batches of commercially prepared standards were highly "contaminated" with cis isomers, as evidenced by secondary absorbance maxima between 320 and 340nm. Crystallization in hexane reverted much of this isomeric mixture to the all-trans form as evidenced by a loss in absorbance in the 320-340nm range (24). Peak identification of all compounds was tentatively confirmed by co-chromatography of standards in these two chromatographic schemes and by the absorption spectra in both the mobile phase and hexane solutions.

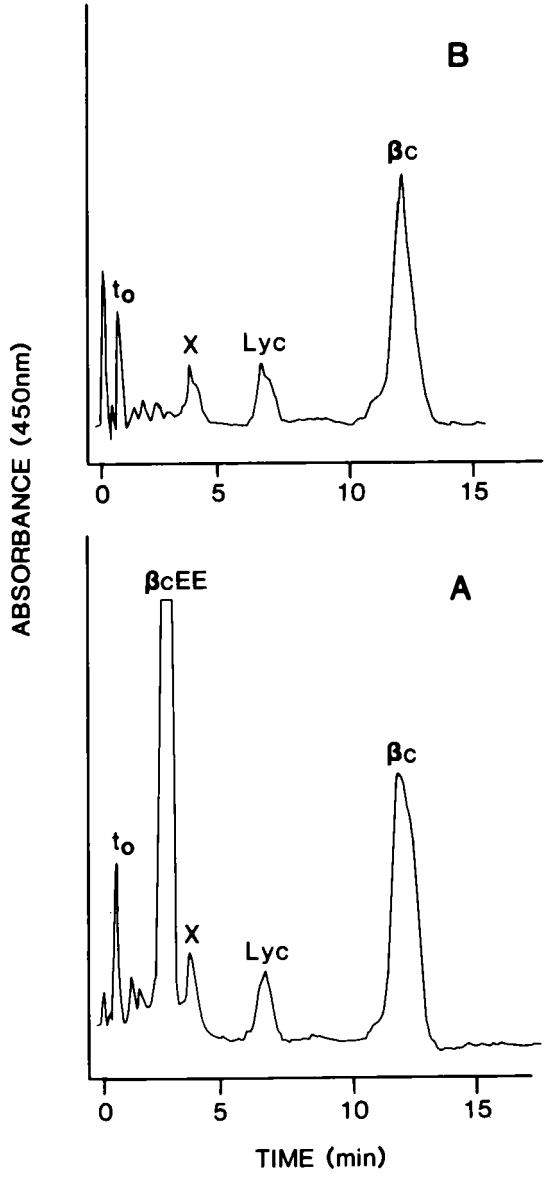

FIGURE 3: A. (lower) HPLC profile of a plasma sample extracted as described in text. The internal standard BcEE was added prior to extraction. B. (upper) HPLC profile of the same plasma sample extracted similarly but with no BcEE added.

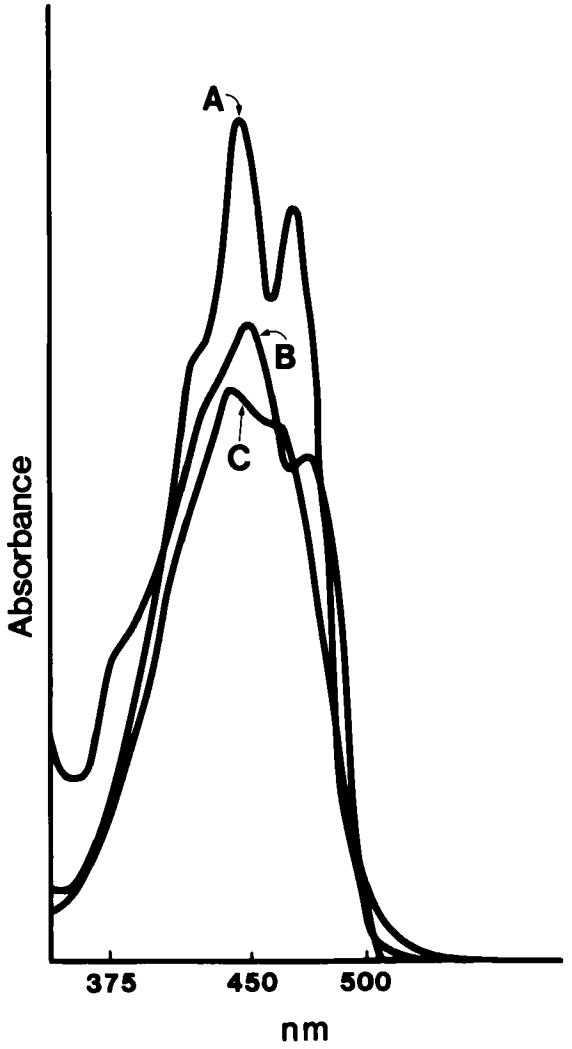

FIGURE 4: Absorption spectra of (A) alpha carotene, (B) beta carotene, and (C) beta-apo-8-carotenoic ethyl ester.

Calibration, Quantification and Recovery Experiments

Calibration curves for alpha and beta carotenes were linear from 20ng/ml to 10ug/ml; lycopene with a poorer absorptivity at 450nm had linear range from 45ng/ml to 8ug/ml. R_2 values for four or five standard solutions injected each day were greater than 0.995; sample analyses were not conducted unless this minimum value was exceeded. A comparison of slopes of calibration curves obtained for six days of analysis yielded a coefficient of variation of 4.78%.

Recoveries of spiked sera and plasma of dog or man ranged 84.80% to 91.30% (Table 1). Extraction efficiencies were significantly improved by the addition of 3% ethanolic ascorbic acid ($p<0.05$). No experiments were attempted however to assess the efficacy of 0.0125% BHT in hexane; this solution was used in all assays. Because there were no significant differences in recovery efficiencies between samples spiked with 1.225ug beta carotene and 2.45ug beta carotene, the means and coefficient of variation represent those of all six replicates. Nor was there a significant difference between percent recoveries obtained for human plasma and that of dog. Similar improvements in recoveries of labile vitamins A and E with these antioxidants were reported by Chow and Omaye (25), but slightly higher concentrations of ascorbic acid and BHT were used.

Plasma Concentrations in Human Volunteers

Plasma beta carotene levels of four subjects taking varying amounts of Solatene daily are presented in upper half of Figure 5. All subjects were consuming their "usual" diets at that time (late winter/early spring) but actual dietary intakes were not monitored. None of the subjects consumed other vitamin/mineral supplements. The investigator ascertained pill compliance by direct observation. The

TABLE 1

Recovery Efficiencies for Beta Carotene in Various Tissues.

	Antioxidant	Percent Recovery	
Sample	Ethanol Phase	X	CV[a]
Plasma, human	0	81.95 } d	11.6/
Plasma, human	3% AA[b]	91.30[c] }	3.67
Sera, human	0	79.97 } d	7.29
Sera, human	3% AA[b]	90.06[c] }	4.08
Plasma, dog	3% AA[b]	84.80[c]	0.92

[a] Coefficient of Variation, %.

[b] Ascorbic Acid.

[c] Values represent that of six replicates, that is the percent recoveries at both spiking levels - 1.225ug and 2.45ug beta carotene.

[d] $p < 0.05$

supplementation regimen is depicted graphically in the lower half of Figure 5 of each volunteer. Excluding the baseline determinations, the contribution of these supplements to plasma levels probably far outweighed that made by diet. Recent evidence from Shekelle et al (26), Willett and coworkers (27) and unpublished observations in this laboratory suggest that typical beta carotene intakes for the American population range from 1.5 to 7.0mg per day. Admittedly, these estimates are crude at best since the beta carotene content of foods were not directly determined but derived from indices based on food groups or partition coefficients. In spite of these limitations, it is likely that the volunteers in the current study consumed from 10 to 60 times as much as that usually obtained from diet. Baseline plasma determinations of three of the four volunteers were considerably higher than the average

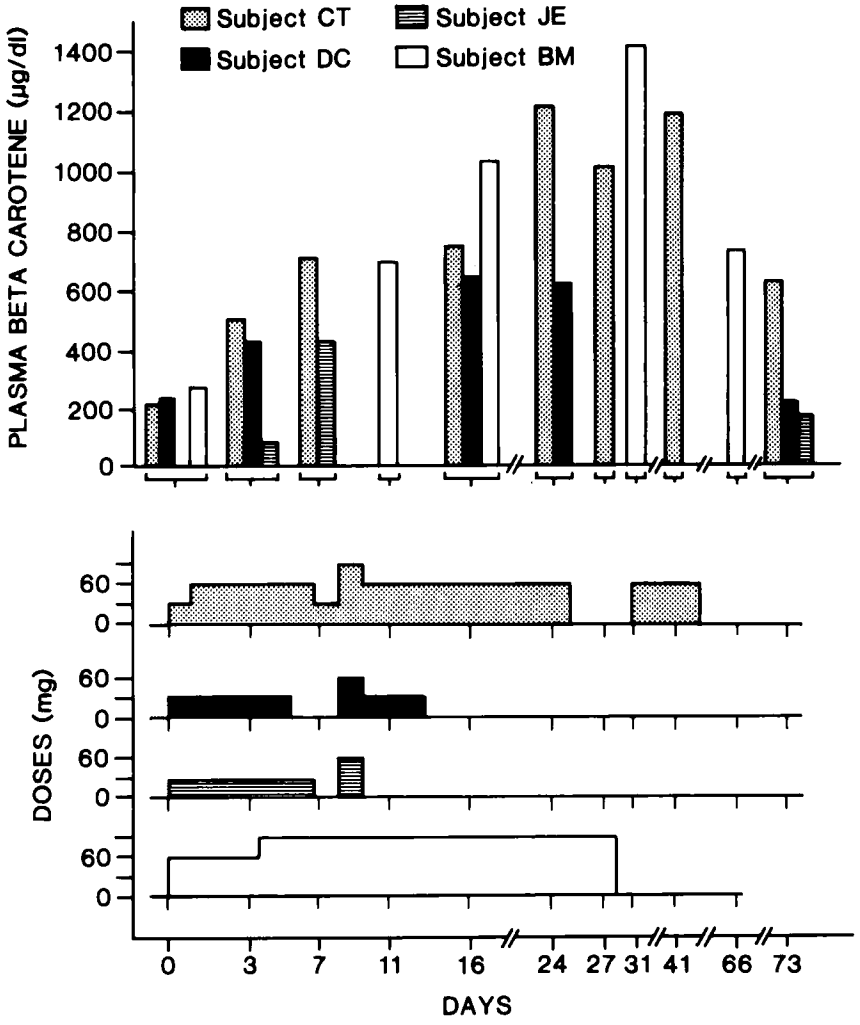

FIGURE 5: Plasma beta carotene levels of four subjects (upper graph) ingesting various amounts of Solatene (lower graph) over time.

beta carotene levels reported by Ross and Parker (28) but well within the "normal" ranges. All subjects exhibited barely detectable lycopene levels (0.51-4.55ug/dl); there was no consistent pattern of change in these levels concomitant with the supplement dose ingested. Detectable alpha carotene levels were observed for only one volunteer, C.T., and this concentration was only observed at one time only. The third subject, J.E., though exhibiting undetectable levels prior to supplementation, later evidenced higher levels that were apparently reponsive to supplementation. A fivefold increment in plasma beta carotene levels was observed from day 3 to day 7. The changes in concentration for the remaining three subjects with 30 or 60mg Solatene administration were approximately three- to fourfold from day 0 to day 16. In subject B.M. who ingested the highest dose (90mg) daily for 24 days, plasma beta carotene levels increased nearly sixfold from day 0 to day 31. The response to dose administration appeared quite rapid for all subjects, although the rate of response seems to be quite different from subject to subject. The actual rate of response is difficult to assess because blood samples were not obtained at earlier time points nor at the same time points with similar doses for subjects. In contrast to the seemingly rapid response in plasma levels with initial doses, the cessation of Solatene therapy was not accompanied by a precipitous drop in plasma levels (days 31-73). By days 66 or 73 (more than a month after supplementation was discontinued) levels were still elevated in three of the four subjects. Plasma levels of subjects B.M. and J.E. measured 45 days and 67 days later, respectively, were still much higher than baseline levels. The relative responsiveness of plasma levels to supplementation has also been examined by Bjornson and associates (29) and Mathews-Roth et al (30). In the former, several patients with diagnosed erythropoietic

protoporphyria ingesting 60 to 120mg Solatene demonstrated a similarly sharp rise in beta carotene levels with the initiation of therapy and more gradual lowering with discontinuation. A direct comparison is not possible here because of differences in patient characteristics, dose levels, time and analytical technique. Willett and coworkers (27) measured total serum carotenoids in 59 health adults consuming only 30mg of Solatene at 0, 8, and 16 weeks. These investigators found that plasma carotenoids nearly tripled by 8 weeks. Again, the study design was considerably different and no determinations were conducted following discontinuation of supplements. While prior literature confirms the basic trend observed in this pilot study, few if any have employed a chromatographic technique that is as specific for beta carotene or as reliable and fast. In future large scale trials in which supplement use or dietary intakes will be monitored by plasma determinations, it is essential that such a technique be available for investigators. Further research is necessary to quantitate the degree of association between dietary intakes and plasma levels using a specific measurement for beta carotene in tissues. These studies are presently ongoing in our laboratories.

ACKNOWLEDGEMENTS

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