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SERUM AND PLASMA β-CAROTENE LEVELS MEASURED WITH AN IMPROVED METHOD OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An isocratic high-performance liquid chromatographic method specifically developed to allow simple and rapid determination of β-carotene concentrations in serum and plasma is reported. Using a method modified from a previously published technique, serum and plasma proteins are denatured by exposure to perchloric acid, and β-carotene is subsequently extracted into an organic matrix consisting of ethyl acetate—tetrahydrofuran (1:1); no evaporation step is required. Separation is achieved using isocratic elution from a reversed-phase C_{18} column with UV detection at 436 nm. Recovery of β-carotene from water and plasma was greater than 98.1%; β-carotene was stable in the extraction matrix for at least 4 h. Three anticoagulants (oxalate, citrate, and EDTA) caused losses of β-carotene; perchloric acid and tetrahydrofuran could also destroy β-carotene under certain conditions. Each run required less than 15 min; within-day coefficient of variation for identical samples averaged 2.3%, between-day coefficient of variation was 4.4% and sensitivity was better than 10 ng/ml. Stability of β-carotene in plasma was also examined. This method permits a simple, rapid, sensitive, precise, and accurate determination of β-carotene using 0.5 ml of serum or heparinized plasma.

INTRODUCTION

In the last few years, data from several sources have suggested that β-carotene, a naturally occurring provitamin of retinol (vitamin A), might have anti-cancer properties. The ability of β-carotene to inhibit tumor formation in animals [1] and the association of higher dietary levels of β-carotene with lower rates of cancer such as lung cancer [2], have led several reviewers to suggest that β-carotene might be a clinically important anti-cancer agent [3, 4]. Because of this type of evidence, large prospective clinical trials of β-carotene

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as a cancer chemopreventive agent are now being performed. We are currently conducting such a study at our institution.

It is therefore important to be able to measure blood levels of β-carotene easily and accurately. Measurement of blood levels of β-carotene by high-performance liquid chromatography (HPLC) has been shown to be faster, more accurate, more precise, and more selective than older methods such as open-column techniques, thin-layer chromatography, and conventional spectrophotometric methods [5–10].

One of the earliest HPLC methods published for the determination of β-carotene [5] involved time-consuming saponification and solvent evaporation steps, which would make routine analysis of the large number of samples obtained in large prospective clinical studies considerably more difficult. Two recently reported methods [9, 10] achieved excellent precision, but also required a solvent evaporation step during the sample clarification process.

In 1983, a precise method [6] for measuring β-carotene in human plasma was reported which did not require a solvent evaporation step. However, this method used an internal standard not commercially available and it failed to separate α- from β-carotene. Moreover, the sensitivity (80 ng/ml), although sufficient for normal subjects, would likely not have been satisfactory for subjects with lower dietary intakes and blood levels of β-carotene. Another method reported during the same year [7] used a commercially available internal standard (retinol acetate) and did not require a solvent evaporation step. However, monitoring both the β-carotene peak and the internal standard peak required two different wavelengths, which would raise detector costs considerably. In addition, within-day precision (measured by the coefficient of variation, C.V.) for three carotenoids was between 6.4% and 13.5%, sensitivity of β-carotene detection was not stated and recovery of β-carotene was only 93.6% from rat serum. Recovery from human samples was not evaluated.

In 1983, Peng et al. [8] published a method for β-carotene determination which appeared to be perhaps the most simple, rapid, and sensitive one. However, several questions about this method arose when we attempted to use it in our laboratory. (1) Did the tetrahydrofuran (THF) used in the extraction step and in the mobile phase need to be preserved to reduce the accumulation of peroxides? (2) Was β-carotene labile in the presence of perchloric acid used to denature plasma proteins? (3) How were the external standards prepared? (4) Why was β-carotene unstable in the organic matrix after extraction? (5) What were the within-day and between-day estimates of precision?

In addition to these specific questions, we raised several questions of a more general nature which had not been adequately addressed to in the literature. Is there a difference between plasma and serum levels? If plasma is assayed, does the anticoagulant used have any effect upon β-carotene levels? How quickly must the blood samples be processed and frozen? What are the optimal conditions for long-term storage, and do repetitive cycles of freezing and thawing result in deterioration of β-carotene?

In this paper we demonstrate that a modification of the method of β-carotene determination published by Peng et al. [8] is simple, precise, and sensitive. We address all the questions about their method outlined above.

Finally, we address the general methodologic issues which must be considered by other investigators who plan to collect blood samples and assay them for β-carotene.

MATERIALS AND METHODS

Reagents

Crystalline β-carotene was the highest grade available, and was purchased from Sigma (St. Louis, MO, U.S.A.). This product had only one peak when subjected to HPLC analysis. Butylated hydroxytoluene (BHT) was purchased from the same source. Acetonitrile and methanol were HPLC grade and purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). Ethyl acetate and THF preserved with BHT were purchased from Burdick & JacksonLabs. (Muskegon, MI, U.S.A.). Ammonium acetate (HPLC grade), crystalline potassium iodide, concentrated hydrochloric acid, and 70% perchloric acid were purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.). Water used for HPLC mobile phase preparation was house-distilled, then passed through a Milli-Q purification system (Millipore, Bedford, MA, U.S.A.). Absolute ethanol was purchased from U.S. Industrial Chemicals (Tuscola, IL, U.S.A.).

Blood samples

Vacutainer glass tubes were used to collect serum and four types of plasma (anticoagulated with lithium heparin, oxalate, citrate, or EDTA) and were obtained from Becton-Dickinson (Rutherford, NJ, U.S.A.). Serum and plasma samples were protected from direct light (sunlight or fluorescent light) and centrifuged at room temperature within 30 min of drawing unless otherwise stated. After separation, the serum or plasma was transferred by a glass pipet to polypropylene freezer tubes (Nunc brand, A.H. Thomas, Philadelphia, PA, U.S.A.) and analyzed immmediately unless otherwise stated. When stored prior to analysis, the tubes were kept at −35°C. When samples were analyzed after freezing, they were thawed to room temperature, vortexed and centrifuged at 1500 *g* for 5 min to remove any cryoprecipitate present.

Plasma samples for determining between-day precision were obtained from the blood bank. Patients undergoing therapeutic phlebotomy had one unit of whole blood collected in a standard plastic blood bank bag, to which sodium heparin had been previously added. After the phlebotomy was completed, the plasma was separated and stored frozen at −35°C. When needed, the plasma was thawed, mixed thoroughly, centrifuged at 1500 *g* to remove any cryoprecipitate, and transferred to 1-ml polypropylene freezer tubes (Sarstedt, Princeton, NJ, U.S.A.). These individual sample tubes were then re-frozen, and thawed each day when needed.

Peroxide assay

A semi-quantitative assay for the presence of peroxides was utilized. Water (2.5 ml) was placed in a glass test tube, to which were added approximately 250 mg crystalline potassium iodide. Concentrated hydrochloric acid (0.5 ml) was added, and the mixture was mixed with a glass rod. The organic solvent to be assayed was added (1.5 ml) and the tube was vortexed for 30 sec. After 5 min

of incubation, absorbance at 510 nm was measured on a spectrophotometer (Varian Instruments, Springfield, NJ, U.S.A.), using a blank solution prepared as above, but with pure ethanol as the organic solvent.

Standard solutions

Crystalline β-carotene was dissolved in 5 ml benzene to which 15 ml hexane were added. This stock solution demonstrated a loss of β-carotene content of approximately 1% per day when stored at 4°C under nitrogen. Serial dilutions of the stock solution in absolute ethanol were prepared daily to approximate concentrations of 7.0, 3.5, and 1.0 μg/ml. Exact concentrations of these solutions were determined by measuring the absorbance of the 3.5 μg/ml solution at 450 nm, using an extinction coefficient in ethanol ($E^{1\%}_{1\,cm}$) of 2375 [11].

Sample clarification

All procedures were performed in a dark room, illuminated with a 25-W incandescent bulb. To clarify human blood samples, 500 μl plasma or serum were pipetted into a 1.5-ml polypropylene microcentrifuge tube (Fisher Scientific); 50 μl of pure ethanol were added and the tube was capped and vortexed for 15 sec. Protein was denatured by the addition of 100 μl of 5% perchloric acid; after vortexing for 30 sec, 500 μl of the extraction solvent, ethyl acetate—THF preserved with BHT (1:1), were added and the tubes were vortexed for 60 sec. All tubes were centrifuged at 13 000 *g* for 1 min on a Fisher Model 235B microcentrifuge. The organic matrix (top yellow layer) was removed by a glass pipet and transferred to a 500-μl polypropylene microcentrifuge tube (Fisher Scientific), and then centrifuged again at 13 000 *g* for 1 min to remove any remaining microparticulate matter. This final organic matrix was then ready for direct injection into the HPLC system.

To generate the daily standard curve, aqueous solutions were spiked with known concentrations of β-carotene and extracted as above with the following differences: (1) 500 μl water instead of plasma were used; (2) 50 μl of an ethanolic solution of β-carotene instead of 50 μl pure ethanol were added; (3) 100 μl of water instead of 100 μl perchloric acid were added.

High-performance liquid chromatography

The HPLC system consisted of a Waters Model 510 dual-piston pump (Waters Assoc., Milford, MA, U.S.A.), an SSI 0.5-μm in-line filter (Rainin Instruments, Woburn, MA, U.S.A.), a Rheodyne Model 7125 injector with a 100-μl loop (Rainin), a Brownlee precolumn (30 × 4.6 mm) packed with 5 μm diameter RP-18 material (Rainin), an Altex Ultrasphere-ODS column (5-μm spherical packing material, 250 × 4.6 mm, Beckman Instruments, Wakefield, MA, U.S.A.), a Beckman Model 160 UV detector equipped with a 436-nm filter, and a one-channel strip-chart recorder (Model D-5119-1, Houston Instruments, Austin, TX, U.S.A.). A reporter—integrator was also used with equivalent results (Model 3390A, Hewlett-Packard, Avondale, PA, U.S.A.).

Detector sensitivity was set at 0.008 a.u.f.s. The mobile phase consisted of acetonitrile—THF (preserved with BHT)—methanol—1% ammonium acetate

(65:25:6:4). The mobile phase was filtered through 0.45-μm nylon filters (Rainin), degassed under vacuum, and pumped at 2.7 ml/min at ambient temperature, generating a back-pressure of 186 bars. Peak identification was confirmed by comparing plasma peak retention times to those of known standards and by demonstrating coelution of plasma peaks and known standards added to the plasma matrix.

Calculations

The height of the β-carotene peak was measured for each run. A standard curve (β-carotene peak height versus β-carotene concentration) was generated each day from the three spiked aqueous standards (100, 350 or 700 ng of β carotene added to 1 ml water). The best-fit linear regression line was calculated using the method of least squares. The correlation coefficient (r) for each standard curve was also calculated; standard curves were not accepted if the value of r was less than 0.998.

In experiments in which recoveries were tested under different conditions, the significance of differences between means was explored using the one-way analysis of variance. When significant differences were observed, they were further investigated using the Student—Newman—Keuls test [12]. Results of duplicate or triplicate determinations were always expressed as mean ± standard deviation (S.D.). Precision was reported as the C.V. of multiple determinations (C.V. = S.D./mean).

RESULTS AND DISCUSSION

Chromatographic conditions

Our HPLC conditions were similar to those used by Peng et al. [8]. However, we modified the composition of our mobile phase in order to minimize retention volume, while optimizing separation of carotene isomers and peak shape. In addition, we noticed that the peroxide content of the THF employed in both the mobile phase and the organic extraction matrix was critical. THF which was not preserved with BHT, even when new, had measurable amounts of peroxides which caused variable, at times greater than 50%, loss of β-carotene during sample clarification and chromatography. The THF preserved with BHT obtained from Burdick & Jackson Labs. had the lowest peroxide content (several brands were tested) and was used for all of our work. We feel it is important that THF used should always be preserved with BHT; in addition, since lots and sources may vary in peroxide content, each bottle should be assayed prior to use. Finally, the HPLC conditions outlined above are compatible with long analytical column life. We changed our guard column every 400 injections, before any changes were noted in back-pressure or retention time. With this precaution, our analytical column remained satisfactory for over 2200 injections (ten months), before changes in peak symmetry and back-pressure required a new column.

Sensitivity and precision

For spiked aqueous solutions subjected to sample clarification, sensitivity was better than 10 ng/ml using 50-μl injections and a detector sensitivity of

0.008 a.u.f.s. (peak height greater than 5 times baseline noise). This sensitivity is as good or better than that reported in earlier studies [6—10], and is certainly well below the range of β-carotene levels seen in our normal subjects, or reported in other studies [6, 9]. Sensitivity could be increased, if necessary, by either increasing the sensitivity of the UV detector (up to 0.001 a.u.f.s.) or by using an injection volume larger than 50 μl. A typical chromatogram is shown in Fig. 1. This human sample contained 427 ng/ml β-carotene. There is near-baseline separation of the two carotene isomers, and this separation is comparable to or better than that demonstrated in earlier reports [6—10].

Within-day precision was calculated every day, since heparinized plasma standards were run each day in duplicate or triplicate. Over a typical one-month period, within-day precision (C.V.) ranged from 0.0% to 6.7%, with an average value of 2.3 ± 1.7%. Over eleven consecutive days, between-day precision was calculated from the daily mean values of these plasma standards. These eleven values (range 162—186 ng/ml) had a mean value of 173.0 ± 7.6 ng/ml, C.V. = 4.4%. Thus our values of within-day and between-day precision (C.V.) compare favorably to the earlier studies cited above.

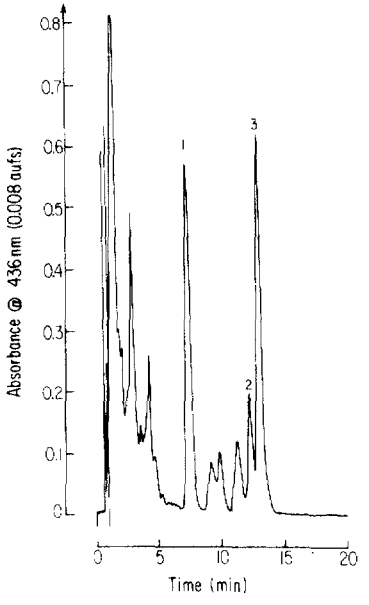

Fig. 1. Chromatogram of a heparanized human plasma sample. HPLC conditions and sample clarification conditions as in text. β-Carotene concentration was calculated to be 427 ng/ml. Peaks: 1 = lycopene; 2 = α-carotene; 3 = β-carotene.

Standard curve

Initially, we prepared our standard curve each day by adding known concentrations of β-carotene to plasma samples and plotting net increase in β-carotene peak height versus amount of β-carotene added. Since it would have been easier to generate daily standard curves from spiked aqueous samples rather than spiked plasma samples, we compared net β-carotene peak heights when water and heparanized plasma samples were spiked with known concentrations of β-carotene. Relative to water (peak height defined as 100%)

recovery of β-carotene from heparanized plasma from two separate subjects was 98.8% and 103.0%, respectively. A full standard curve (y = β-carotene peak height at 0.008 a.u.f.s., x = β-carotene concentration in μg/ml) was prepared from the plasma of a third subject with the best-fit line being $y = 0.489x + 0.006$ ($r = 0.999$). The standard curve prepared using spiked water was $y = 0.493x + 0.002$ ($r = 0.999$).

After demonstrating equal recovery of β-carotene from spiked plasma and aqueous samples, we sought to quantitate absolute recovery of β-carotene from spiked aqueous samples. When β-carotene was added to water to a final concentration of 350 ng/ml, the absorbance of this aqueous solution was 0.260 ± 0.000 in duplicate samples. After both samples were extracted using the usual sample clarification scheme, the residual absorbance in the lower aqueous layer was 0.005 ± 0.000. Thus, at least 98.1% of the β-carotene added to the aqueous layer was removed during the usual extraction process.

We generated a standard curve each day from three aqueous samples spiked with β-carotene (final concentrations 100, 350, and 700 ng/ml) and carried through the entire sample clarification process. This seemed optimal since the recovery of β-carotene from plasma and water was equal; the recovery from water was nearly 100%, and a standard curve so generated would likely alert us to any inter-current difficulties with the entire sample clarification process.

Stability of β-carotene during sample clarification

To 500-μl aqueous samples β-carotene had been added with or without 5% perchloric acid. The mixtures were incubated in the dark at room temperature for 1 or 15 min before the organic matrix was added. Clarification was completed, and the samples were injected immediately into the HPLC system. After injection, some of the organic matrix samples were kept at room temperature in the dark for 3.5 h, and then injected again. The results are summarized in Table I.

With the recovery of β-carotene from water (in the absence of perchloric acid) defined as 100%, it is apparent that incubation for 15 min in the absence of

TABLE I

EFFECTS UPON β-CAROTENE PEAK HEIGHT OF DIFFERENT CONDITIONS DURING SAMPLE CLARIFICATION AND OF PROLONGED STORAGE OF THE ORGANIC MATRIX PRIOR TO INJECTION

Each number is the mean of n determinations and expressed in percent. Each incubation (prior to the addition of the organic matrix) was for either 1 or 15 min. Injection into the HPLC system was either immediately (0 h) or 3.5 h following extraction. Those samples defined as being 100% are underlined.

Sample	1-min Incubation				15-min Incubation	
	0 h	n	3.5 h	n	0 h	n
Water—water	100.0	3	96.3	2	100.0	2
Water—acid	98.9	2	64.5*	2	94.0	2
Plasma—acid	100.0	11	102.2	11	100.4	2

*$p < 0.001$.

perchloric acid or for 1 min in the presence of perchloric acid has no effect upon the recovery of β-carotene. Incubation for 15 min in the presence of perchloric acid caused a small, statistically non-significant, decrease in recovery of β-carotene. When plasma samples without exogenous β-carotene were incubated for 1 min (defined as 100% recovery) or 15 min prior to organic extraction, there was no decrease in recovery of β-carotene. Thus it appeared that β-carotene was stable for 15 min in aqueous solution or for 15 min in plasma treated with perchloric acid. However, β-carotene in aqueous solution was slightly susceptible to 15-min exposure to perchloric acid. In fact, this loss of β-carotene peak height was accompanied by the development of several small, new peaks with retention times less than that of β-carotene and with the development of a new peak occurring on the descending shoulder of the β-carotene peak.

Since our aqueous solutions are extracted promptly in the absence of perchloric acid and since our plasma samples are extracted promptly in the presence of perchloric acid, we felt that there was no demonstration of worrisome acid-induced loss of β-carotene peak height during the clarification process.

Stability of β-carotene in the organic matrix

Although sample clarification can be accomplished promptly without prolonged incubations, we were concerned that the β-carotene extracted into the organic phase might not be stable. Samples were extracted batchwise, and occasionally a sample would have to stand for 15—45 min following extraction before injection onto the HPLC system. Peng et al. [8] reported that samples were stable for 1 h or less after extraction, using a similar extraction scheme. As seen in Table I, when samples were extracted promptly (1-min incubations) but the organic matrix was held in the dark at room temperature for 3.5 h rather than injected immediately, there were variable effects upon β-carotene

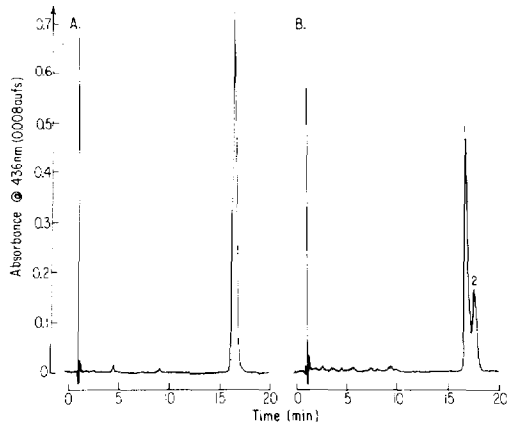

Fig. 2. (A) Chromatogram of β-carotene (1) after extraction from aqueous matrix with exposure to perchloric acid for 1 min, immediate extraction, and immediate injection (peak height 98.9% of control height). (B) Injection of the same organic matrix after 3.5-h incubation in the dark at 22° C. β-Carotene peak height (1) is now 64.5% of control height, possible degradation product (peak 2) is also seen.

recovery. Aqueous samples processed without perchloric acid and plasma samples processed with perchloric acid demonstrated no loss of β-carotene peak height despite the 3.5-h delay prior to injection. However, β-carotene in an aqueous matrix, incubated for 1 min in the presence of perchloric acid, then extracted and held for 3.5 h prior to injection, demonstrated a pronounced loss of β-carotene peak height. This loss of peak height was accompanied by the development of several new peaks on the chromatogram and the development of a large shoulder of the β-carotene peak itself (see Fig. 2).

We believe that this loss of β-carotene, associated with the development of new peaks, represents degradation or isomerization of the parent molecule. β-Carotene is unstable because of its conjugated system of double bonds; it is destroyed or altered by acids, particularly in the presence of light, and it is easily oxidized, leading to epoxide formation and chain cleavage [13]. In addition, we had previously shown that exposure of retinol to perchloric acid during a plasma clarification process resulted in unpredictable but definite losses of retinol and retinol acetate, which led to sub-optimal precision [14]. Perhaps the reason why we found β-carotene to be relatively more stable during sample clarification and in the organic matrix than did Peng's group [8] may be that we used only THF preserved with BHT and we assayed each lot of THF to make sure that the peroxide content was acceptably low. For example, THF preserved with BHT, when subjected to our peroxide assay, produced a yellow color similar to that obtained when pure ethanol was assayed; the absorbance at 510 nm was approximately 0.015 relative to the blank solution prepared with ethanol. When unpreserved or old THF was assayed, the assay produced a reddish-brown color with an absorbance as high as 1.81.

Stability of β-carotene in plasma

Heparanized plasma from four subjects was analyzed (in duplicate) immediately after venipuncture, and again after the plasma had been exposed to laboratory levels of fluorescent light and room temperature for 4 h. After such treatment, β-carotene peak heights were 104.6 ± 5.6% that of the immediately assayed samples. Therefore, it appeared that no special collection procedures (chilling samples, protecting from light) were required as long as samples were centrifuged and assayed (or frozen) within 4 h of venipuncture. Previous authors [8, 15] have also observed that β-carotene in serum or plasma is stable under these conditions for up to 24 h.

Differences between serum and plasma levels

For each of three subjects, blood was obtained from a single venipuncture and transferred to Vacutainer glass tubes containing no anticoagulant (for serum) or one of four commonly used anticoagulants (heparin, EDTA, oxalate, or citrate). After 1 h (to allow clot retraction to occur), the blood samples were centrifuged and the serum or plasma was separated. Samples were clarified, and injected onto the HPLC system within 30 min. Results are summarized in Table II.

Levels of β-carotene in each subject were highest in serum, with levels in heparanized plasma being slightly (but significantly) less. This may reflect

TABLE II

β-CAROTENE LEVELS IN FOUR TYPES OF PLASMA, RELATIVE TO SERUM LEVELS, IN THREE HEALTHY SUBJECTS

All values (in percent) are expressed as mean ± S.D. of duplicate determinations. For each subject, levels in serum were defined as 100%. *p* Value reflects significance of difference between mean plasma levels and mean serum level.

Matrix	Subject A	Subject B	Subject C	Group mean	*p* Value
Serum	100.0 ± 0.6	100.0 ± 5.7	100.0 ± 0.7	100.0	
Heparin	95.7 ± 3.2	98.0 ± 2.0	93.1 ± 0.8	95.6 ± 2.5	<0.05
EDTA	91.8 ± 0.0	92.5 ± 0.2	90.1 ± 0.8	91.5 ± 1.2	<0.01
Oxalate	78.0 ± 0.4	79.2 ± 2.0	76.9 ± 0.3	78.0 ± 1.2	<0.01
Citrate	77.5 ± 0.3	83.4 ± 1.3	75.5 ± 0.8	78.8 ± 4.1	<0.01

the greater protein content of plasma relative to serum. However, such an explanation would not explain the fact that when EDTA, oxalate, or citrate were used as anticoagulants, the loss of β-carotene was even greater. These findings were quite similar to previously reported effects of these anticoagulants upon plasma levels of retinol [14, 16]. While the mechanism of this apparent loss of retinol and β-carotene is not clear, it is possible that EDTA, oxalate, and citrate — all acids — may catalyze reactions resulting in the isomerization or oxidation of retinol and β-carotene. Thus, we suggest that either serum or heparanized plasma be collected when blood levels of β-carotene are to be measured.

Storage conditions for plasma samples

We investigated the effects of multiple freeze—thaw cycles on plasma β-carotene levels by obtaining plasma from one subject, freezing it, storing it at −35°C, and carrying it through sequential freeze—thaw cycles each day for seven days. Plasma levels (all done in duplicate) after 1, 2, 3, 5, 6, and 7 such cycles were 167, 175, 175, 153, 176, and 169 ng/ml. Thus, sample tubes may be thawed and refrozen multiple times without affecting β-carotene levels. This observation supports similar findings previously reported [7].

A related issue concerns optimal long-term storage conditions for blood samples. As shown in Table III, we found that plasma from one subject had

TABLE III

EFFECT OF STORAGE AT DIFFERENT TEMPERATURES UPON β-CAROTENE LEVELS IN HEPARANIZED PLASMA

Baseline levels were measured nine times over one week; levels at six months were measured in duplicate. The *p* value reflects significance of difference between means at six months relative to the mean value of the baseline sample.

Storage conditions	β-Carotene levels (mean ± S.D., ng/ml)	*p* Value
Initial sample	167.7 ± 7.3	
Six months at −7°C	154.5 ± 2.1	<0.025
Six months at −35°C	175.0 ± 7.1	>0.20
Six months at −70°C	174.0 ± 0.0	>0.20

stable β-carotene levels over a six-month period whether stored at −35°C or −70°C. Samples stored at −7°C demonstrated a significant loss of β-carotene after six months. Previously reported studies had shown that total carotenoids in plasma stored at −20°C for ten years are reduced by about 97%; in plasma stored at −70°C for one year, total carotenoid values were essentially unchanged [15]. It would seem to be prudent to store serum and plasma at either of the lower temperatures and assay samples for β-carotene as soon as possible; delays of up to six months seem to be acceptable. Optimal storage conditions for periods greater than six months are being investigated.

Normal plasma β-carotene levels

We have analyzed plasma levels of β-carotene from many patients with specific diseases, as well as from seventeen healthy volunteers who were medical school employees, not taking any vitamin supplements containing β-carotene. These volunteers were eleven women and six men, aged 24–55 years. Their range of β-carotene levels was 91–428 ng/ml (mean ± S.D. 274 ± 103 ng/ml).

Two prior studies used HPLC methods [6, 9] to measure serum or plasma levels of β-carotene in normal subjects. One study [6] reported a range of 150–930 ng/ml (mean ± S.D. 330 ± 205 ng/ml) in twenty normal volunteers. A second study [9] reported mean levels of 331 ± 191 ng/ml in eight normal men, and 351 ± 164 ng/ml in eighteen normal women. Both of these studies compared β-carotene levels determined by HPLC with those determined by conventional spectrophotometric methods. In each case, the level determined by HPLC was lower. Thus, the range of plasma β-carotene levels in normal subjects as determined by HPLC methods is beginning to be ascertained, and it appears to be much lower than the reported normal range for total carotenoids.

Further studies using BHT as a preservative

After the above experiments were completed, we sought to slow the rate of loss of β-carotene in the benzene–hexane stock solution. Even though it was stored at 4°C under nitrogen, this solution lost β-carotene at a rate of approximately 1% per day. In an attempt to slow this rate of loss, we added BHT to the hexane, such that the final concentration of BHT in the benzene–hexane solution was 125 μg/ml. Under these conditions, there was no loss of β-carotene over thirty days. This observation, combined with a recent report of BHT being used to stabilize labile compounds during the clarification process [17] stimulated us to investigate the use of BHT during our sample clarification process.

Plasma from ten subjects was analyzed using two methods. The first method was as outlined in Materials and methods. The second method was identical, except that the ethanol added just prior to the addition of perchloric acid contained 1625 μg/ml BHT. The final concentration of BHT in the mixture of plasma, ethanol, and perchloric acid was 125 μg/ml. For each subject, the amount of β-carotene measured using BHT during clarification was defined as 100%. Relative to this, the amount of β-carotene measured without using BHT ranged from 91% to 103%, mean 97.7 ± 4.0% (n = 10). While this difference between group means was not significant (p = 0.10), the use of BHT did make a

clinically important difference in several subjects, increasing the recovery of β-carotene. We therefore decided to use BHT in our sample clarification process for all subjects.

Finally, we also investigated whether the use of BHT affected the results of the clarification of the aqueous standards. Standard solutions were clarified in triplicate, using ethanol with or without BHT, as above. Those samples extracted without BHT yielded β-carotene values which on average were 98.4% of those obtained with BHT. Although this difference was not significant, we continued to use BHT during the processing of the aqueous standard solutions, as well as during the processing of plasma samples.

In conclusion, this report describes a method for determining β-carotene levels in human serum or heparanized plasma which is simple, rapid, precise, and sensitive. The absence of a solvent evaporation step makes this method more attractive for groups analyzing the large number of samples collected during large-population studies. Methodologic problems seen with earlier methods (possible loss of β-carotene due to exposure to perchloric acid, peroxides in THF, or heat during solvent evaporation; differences between β-carotene levels in different types of blood samples) were investigated. Preliminary data on optimal sample handling conditions, storage conditions, and values in normal subject are reported. As more clinical studies are begun which require the determination of blood levels of β-carotene, we anticipate that the observations reported here may be of use to clinical investigators and clinical chemists alike.

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