Resolution and Quantitation of the Predominant Geometric β-Carotene Isomers Present in Human Serum Using Normal-Phase HPLC†

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An isocratic high-performance liquid chromatography (HPLC) method completely resolving *all-trans*-, 9-*cis*-, and 13-*cis*-β-carotenes in human serum was developed. The chromatographic separation was achieved on a calcium hydroxide stationary phase following sample extraction and fractionation on fully activated neutral alumina. A combination of *p*-methylanisole and acetone was used to modify the hexane (1:1:98) mobile phase, which improved the tailing of later eluting peaks generally associated with chromatographic separations on calcium hydroxide. Sample fractionation on alumina was used to remove components present in serum that interfered with the HPLC separation of β-carotene isomers. The average analytical recovery was greater than 90% for *all-trans*-, 9-*cis*-, and 13-*cis*-β-carotenes, and control studies demonstrated that minimal isomerization occurred during extraction and analysis of serum samples. Analysis of pooled serum samples indicated that 13-*cis*-β-carotene was the secondary β-carotene isomer present in human serum with the *all-trans* form predominating.

Keywords: *Calcium hydroxide; β-carotene cis isomers; human serum*

INTRODUCTION

Carotenoids are epidemiologically linked with the prevention of several degenerative human health conditions, including cancer and heart disease (Olson, 1992). Mechanistic conjecture concerning these correlations presents a wide range of roles that carotenoids may play at the cellular level, such as intervention during deleterious oxidation reactions (Burton and Ingold, 1984) or enhancement of intercellular gap junctional communication (Bertram et al., 1991). Certain carotenoids, due to their individual physicochemical characteristics, may be more effective than others in specific situations, e.g. singlet oxygen quenching (Di Mascio et al., 1989; Miki et al., 1991), emphasizing the necessity of accurately identifying individual carotenoids present in various biological tissues.

Because of their purported attributes, the identification and quantitation of carotenoids present in human serum have been the subject of a great deal of analytical research and continue to be vigorously pursued (Handelman et al., 1992; Khachik et al., 1992; Stahl et al., 1992). To date, 18 individual carotenoids have been identified in human serum using HPLC (Khachik et al., 1992). In addition to the analysis of structurally unique carotenoids, several investigators have tentatively identified individual members of carotenoid geometric isomer sets using reversed-phase HPLC with either isocratic (Rushin et al., 1990; Stahl et al., 1992) or gradient elution (Handelman et al., 1992). The formation of geometric β-carotene isomers (Figure 1) during common food processing procedures (Sweeney and Marsh, 1971; Chandler and Schwartz, 1988) suggests that physiologically significant levels are consumed by humans. Moreover, certain individual geometric carotenoid isomers possess physicochemical characteristics which are markedly different from other members of their set (Zechmeister, 1962a; Ben-Amotz, 1989), implying different biological functions may be possible for each.

Little is known about the absorption and transport of individual geometric β-carotene isomers in humans. The first priority in determining the fate of these isomers *in vivo* during human metabolism initially must be the development of analytical methods that can both resolve and quantify individual β-carotene isomers present in biologically relevant tissue matrixes such as human serum and organs. The purpose of the following work was to develop one such method using a rapid sample preparation and chromatographic procedure that resolves the predominant geometric isomers of β-carotene present in human serum, thereby facilitating quantitation of these isomers.

MATERIALS AND METHODS

Pooled serum samples (20 mL) obtained weekly from the North Carolina Department of Public Health in Raleigh, NC, were transported on ice, protected from light. Upon arrival at the laboratory the samples were stored at −20 °C until assay. Solvents were reagent grade (Fisher Scientific, Raleigh, NC) and, for HPLC procedures, degassed by filtration under reduced pressure through a 0.45 μm pore-size filter. All sensitive procedures were performed under subdued yellow lighting.

Preparation of Standards. Standards for *all-trans*-, 9-*cis*-, and 13-*cis*-β-carotene were prepared from heat-processed spinach as previously described (Rushin et al., 1990). Crystalline 15-*cis*-β-carotene was a gift from Hoffman-La Roche (Nutley, NJ) and used without further purification. Crystalline α-carotene was obtained from Sigma Chemical Co. (St. Louis, MO) and purified by elution with hexane on deactivated alumina. Standards were identified by elution order on calcium hydroxide and electronic absorption data. These results were identical to previous work in this laboratory confirming the identity of the *all-trans*, 9-*cis*, and 13-*cis*-β-carotene isomers with ^{1}H NMR (Rushin et al., 1990). The 15-*cis* and di-*cis* β-carotene isomers noted in the separation of

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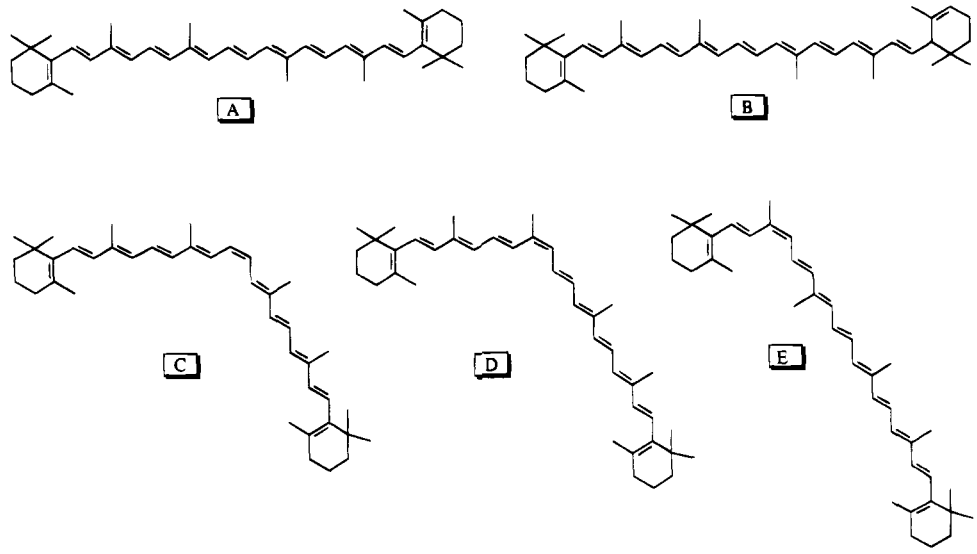

Figure 1. Geometrical conformation of the structural isomers *all-trans*-β-carotene (A) and α-carotene (B) and three mono-*cis* isomers of *all-trans*-β-carotene and 15-*cis*, (C), 13-*cis* (D), and 9-*cis* (E) β-carotene.

isomerized *all-trans*-β-carotene were tentatively identified by chromatographic retention behavior, electronic absorption spectra, comparison with previously published identification data (Tsukida, 1992) and, in the case of 15-*cis*-β-carotene, cochromatography with an authentic standard. A mixture of β-carotene isomers was obtained from processed spinach by chromatographing a hexane extract on fully activated neutral alumina weakened with 4% water (Britton, 1985) and eluting the carotene band with 1.0% acetone in hexane. This mixture contained a detectable quantity of *all-trans*-α-carotene in addition to the geometric β-carotenes.

Serum Sample Preparation. Serum samples were extracted according to Rushin et al. (1990). Two milliliters of serum was placed in a 15 mL centrifuge tube along with 2 mL of absolute ethanol. This mixture was vortexed for 30 s, followed by the addition of 2 mL of hexane to each tube, after which they were vortexed for an additional 60 s and centrifuged at approximately 500*g* for 5 min. The hexane layer was then removed by pipet, and three aliquots of hexane (approximately 1.5 mL) were gently added to the top of the solution and subsequently removed to ensure complete removal of the carotene present in the hexane layer of the extract. The hexane aliquots were pooled and dried over anhydrous sodium sulfate. The extract was then fractionated according to polarity on a Waters Sep-Pak Classic containing fully activated neutral alumina (Waters, Inc., Milford, MA) using the following elution gradient:

elution solvent	volume (mL)	hexane (%)	acetone (%)
1	6.0	100	0
2	2.0	97	3
3	3.5	90	10

The 10% acetone in hexane fraction was collected and evaporated to dryness under a stream of nitrogen and stored at −20 °C until HPLC analysis (within 48 h).

HPLC Instrumentation and Sample Analysis Conditions. The HPLC system consisted of a Model 510 pump controlled by an automated gradient controller, a Model U6K injector, and a Model 990 photodiode array detector (Waters) equipped with an Powermate SX/20 series computer (NEC Information Systems, Inc., Boxborough, MA) to obtain electronic absorption data. Quantitative detection of β-carotene isomers present in serum samples was achieved using an SM 95 UVIS detector (ANSPEC, Ann Arbor, MI) coupled with a Shimadzu CR601 Chromatopac integrator (Kyoto, Japan). All chromatograms were monitored at 410 nm.

all-trans-, 9-*cis*-, and 13-*cis*-β-carotenes were purified using a semipreparatory calcium hydroxide column (500 mm × 9.4 mm) and an isocratic eluent of 0.8% acetone in hexane flowing at 2.5 mL/min. Quantitative analysis was achieved with an isocratic eluent consisting of *p*-methylanisole/acetone/hexane (1:1:98) using a calcium hydroxide column (250 mm × 4.6 mm) and the following flow rate gradient:

time (min)	flow rate (mL/min)
0	0.6
12.5	0.6
13.0	0.9

The increase in flow rate from 12.5 to 13.0 min was linear, and the flow rate was returned to 0.6 mL/min upon completion of the chromatogram. Acetone was used as the sole modifier when obtaining electronic absorption data due to interference in the UV region of the spectrum by *p*-methylanisole (Schwartz and Patroni-Killam, 1985). After approximately 30 sample injections, the column was washed with 40% acetone in hexane to remove adsorbed lipid material and maintain column performance.

It should be noted here that calcium hydroxide provided from different sources or different lots of material from the same source may vary in activity, thus the modifier composition of the developing solvent may have to be increased or decreased to achieve the desired separation and analysis time. Once a batch of calcium hydroxide is determined to be satisfactory for the chromatographic application, it is recommended that the investigator obtain a surplus of material from this lot. The injection solvent for all standards and samples was hexane. Serum extracts were dissolved in 100 μL of hexane, and 10 μL was injected into the HPLC system.

The analytical column used for this analysis was packed with calcium hydroxide (Aldrich Chemical Co., St. Louis, MO) sieved through 500 mesh and equilibrated to 44% relative humidity in a K_2CO_3 saturated chamber for 48 h. Six grams of this calcium hydroxide was suspended in 50 mL of 0.5% acetone in hexane and sonicated for 10 min. Slurry packing of the column was then accomplished using a column packing reservoir (Scientific Systems, Inc., PA) pressurized to 2600 psi using a Haskel air-driven fluid pump (Haskel, Inc., Burbank, CA). Approximately 100 mL of packing solvent was allowed to percolate through the column while under packing pressure.

Quantitation of β-Carotene Isomers. Standard concentrations were calculated from published absorptivity values for these isomers in hexane (Stitt et al., 1951) using a Shimadzu UV-240 recording spectrophotometer (Kyoto, Japan). Quantification of β-carotene isomers present in serum was accomplished using an external calibration curve. Peak area integration of *all-trans*-β-carotene monitored at 410 nm was determined over a range of 1.98−89.2 pmol at six different points. The response was linear (r = 0.9970). Detection at 410 nm has been determined in this laboratory to approximate the isosbestic point for *all-trans* and the mono-*cis* β-carotene

isomers (O'Neil et al., 1991). Thus, detectable 9-*cis*-, 13-*cis*-, and *all-trans*-β carotene isomers could be quantified using this calibration curve. The detection limit for this assay was 0.90 pmol of β-carotene. All analyses were carried out in duplicate.

Identification of β-Carotene Isomers in Serum. Assignment of the *all-trans*-, 9-*cis*-, and 13-*cis*-β-carotenes was done by comparison of analyte retention behavior with that of the standard compounds on alumina, polymeric C_{18} and calcium hydroxide supports and cochromatography with the standard compounds on calcium hydroxide. In addition, electronic absorption data were collected using the photodiode array detector, and these results were consistent with published absorption characteristics.

Recovery Studies. A known amount of either 9-*cis* (n = 4), 13-*cis* (n = 4), or *all-trans* (n = 4) β-carotene in hexane was added to 2 mL serum aliquots. These were then extracted and analyzed along with 2 mL serum aliquots (from the same pooled serum sample) without added carotene (n = 3). The sample extracts were analyzed in duplicate by HPLC. Recovery was greater than 90% (standard deviation = 7.8%) for 9-*cis*-, 13-*cis*-, and *all-trans*-β-carotene isomers. Replicate samples analyzed at later times (7, 8, and 9 days) yielded total β-carotene values equivalent to the control samples initially analyzed; however, artifactual formation of 9-*cis*-β-carotene was observed in the 9-day sample, suggesting that the storage stability of the β-carotene isomers present in serum extracts (evaporated, sealed under nitrogen and frozen) is finite.

RESULTS AND DISCUSSION

Normal-phase HPLC using calcium hydroxide (Tsukida, 1992) or alumina (Vecchi et al., 1981) as the stationary phase has demonstrated the greatest ability to resolve geometric β-carotene isomers when compared with reversed-phase HPLC (Craft et al., 1990; O'Neil et al., 1991). Therefore, a chromatographic method employing a calcium hydroxide column, which is less fastidious with regard to moisture content and temperature than alumina for isomeric β-carotene separations (Tsukida, 1992), was targeted for development of a human serum assay.

The mobile phase modifier typically chosen for HPLC employing a calcium hydroxide stationary phase to separate geometric β-carotenes is acetone (Tsukida, 1992; O'Neil and Schwartz, 1992). However, the use of acetone during the present study was observed to cause peak tailing, especially of the 9-*cis* isomer eluting after *all-trans*-β-carotene, and inconsistent resolution of the compounds eluting in the 13-*cis* region of the chromatogram (Figure 2b). *p*-Methylanisole has previously been shown to yield narrower analyte bands than acetone when used to modify hexane based developing solvents for either open column or thin layer chromatography employing calcium hydroxide stationary phases (Bickoff, 1948; Schwartz and Patroni-Killam, 1985). Therefore, *p*-methylanisole was tested during development of the HPLC method used in this study. A similar favorable effect on the chromatographic behavior of β-carotene isomers was observed when *p*-methylanisole was incorporated into the mobile phase replacing acetone as a modifier (Figure 2a). Unfortunately, the use of *p*-methylanisole for serum analysis resulted in coelution of α-carotene and 13-*cis*-β-carotene. The addition of equal volumes of acetone and *p*-methylanisole was an effective compromise which affected sufficient resolution between α-carotene and 13-*cis*-β-carotene (Figure 2c) and maintained better peak shape than acetone alone.

This laboratory has previously attempted to apply calcium hydroxide to the resolution of β-carotene isomers present in human serum; however, neither unsaponified nor saponified extracts were effectively chromatographed (Rushin et al., 1990). For the present study, a rapid sample preparation method was developed using cartridges packed with fully activated alumina to concentrate the carotene fraction of the extract and to remove serum components deleterious to the chromatography on calcium hydroxide stationary phase. Chromatograms of different pooled serum samples without added β-carotenes or spiked with either 9-*cis*-, 13-*cis*-, or 15-*cis*-β-carotene isomers are shown in Figure 3.

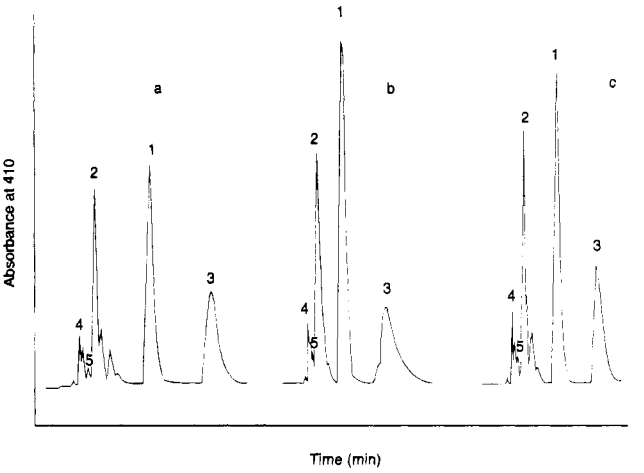

Figure 2. HPLC separation of geometric β-carotene isomers using a calcium hydroxide stationary phase and a mobile phase of hexane modified with either (a) 2.0% *p*-methylanisole, (b) 2.0% acetone, or (c) 1.0% *p*-methylanisole and 1.0% acetone. Peak 1 is *all-trans*-β-carotene, peak 2 is 13-*cis*-β-carotene, peak 3 is 9-*cis*- β-carotene, peak 4 is 15-*cis*-β-carotene, and peak 5 is α-carotene.

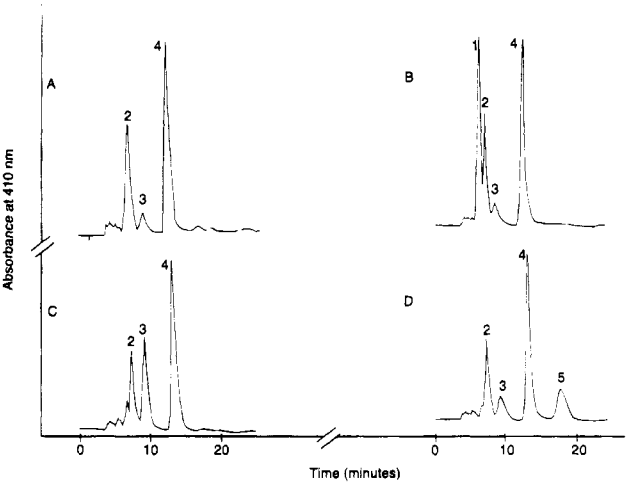

Figure 3. HPLC chromatograms of (A) unsupplemented human serum extract, (B) human serum extract supplemented with 15-*cis*-β-carotene, (C) human serum extract supplemented with 13-*cis*-β-carotene, (D) human serum extract supplemented with 9-*cis*-β-carotene. Peak 1 is 15-*cis*-β-carotene, peak 2 is α-carotene, peak 3 is 13-*cis*-β-carotene, peak 4 is all-*trans*-β-carotene and peak 5 is 9-*cis*-β-carotene. Chromatographic conditions are as described in the text.

Use of the sample preparative step incorporates one additional step to previously published serum extraction and analysis procedures (Rushin et al., 1990). The time required for this step is minimal (less than 15.0 min), and the chromatographic efficiency markedly benefits. Recovery experiments for 9-*cis*-, 13-*cis*-, and *all-trans*-β-carotene demonstrated satisfactory recovery of each analyte although slightly lower than those previously reported (Rushin et al., 1990). Results show no detectable artifactual isomerization until more than 8 days

Table 1. Concentration of Geometrical β-Carotene Isomers in Pooled Human Serum Samples

	mean ± SD,[a] nmol/L		contribution, %	
pooled sample	*all-trans*	13-*cis*	*all-trans*	13-*cis*
1	102 ± 3	7 ± 1	93.2	6.8
2	95 ± 4	–[b]	100.0	–[b]
3	109 ± 1	9 ± 2	91.8	8.2
4	113 ± 1	6 ± 1	95.0	5.0
5	91 ± 1	2 ± 1	97.9	2.1
6	123 ± 1	7 ± 1	94.6	5.4
7	50 ± 1	–[b]	100.0	–[b]
mean	98	4	96.1	3.9

[a] 9-*cis*-β-Carotene was below the detection limit in each of the seven pooled samples. [b] 13-*cis*-β-Carotene was below the detection limit for these pooled sample.

had elapsed between the time of extraction and HPLC analysis. This is consistent with reports on the lack of artifactual isomerization of β-carotene observed when using the extraction (Rushin et al., 1990) and sample concentration (Zechmeister, 1962b) protocol employed in this method.

Complete solubilization of the extract and subsequent miscibility in the HPLC mobile phase is another critical consideration when developing a method for the separation of compounds present in a biological extract (Khachik et al., 1988). Such considerations are often problematic when reversed-phase HPLC is the method of choice due to the more hydrophilic nature required of the mobile phase, particularly when greater efficiency is required for difficult separations such as that of the β-carotene isomers (Jensen et al., 1982; Craft, 1991). The adverse effects observed as a result of choosing an inappropriate injection solvent in reversed-phase HPLC have been documented (Khachik et al., 1988; Scott, 1992). Elution solvents characteristic of normal-phase chromatography ensure complete solubility of lipophilic analytes throughout chromatographic analyses; thus, lipophilic sample solubility problems often encountered in reversed-phase HPLC do not occur during normal-phase HPLC.

Quantitation of the β-carotene isomers present in the serum sample used as controls for recovery studies yielded data which are in accordance with serum concentrations of total β-carotene (Barua and Furr, 1992; Stahl et al., 1993a) and recent reports which indicate that 13-*cis*-β-carotene is present at approximately 5% of the *all-trans* isomer while the 9- and 15-*cis* isomers are generally present in human serum at no more than trace levels (Table 1) (Stahl et al., 1992, 1993a). This observation was also noted when ingestion of 9-*cis*-β-carotene in human subjects was unusually high (Stahl et al., 1993b). In contrast, analysis of certain solid tissues obtained from human subjects indicates the presence of significant quantities of 9-*cis*-β-carotene (Stahl et al., 1992, 1993a). Zechmeister (1962c) has previously noted that certain *cis–trans* isomeric carotenoids may undergo "bio-stereoisomerization" in the rat and chick, and the transformation of 9-*cis*-β-carotene to the *all-trans* form in substantial amounts in rats fed the 9-*cis* isomer exclusively has been reported (Kemmerer and Fraps, 1945). However, a biological mechanism has yet to be elucidated for these bio-stereoisomerizations. Reports suggesting that certain β-carotene isomers may be preferentially absorbed (Ben-Amotz, 1989) or preferentially accumulated and stored in certain tissues (Ben-Amotz, 1989; Stahl et al., 1992) or may possess biological function unique to their *Z*-configuration (Sies et al., 1992) are certainly intriguing and should be investigated further. Indeed, recently elucidated biological functions of the geometric retinoid isomer, 9-cis retinoic acid (Norman, 1992; Franceschi, 1993), emphasize the possibility that structurally related carotene isomers may have critical, although currently unknown, roles in nutritional chemistry.

The ability of calcium hydroxide to resolve individual β-carotene isomers may be able to provide information supplementary to that of current reversed-phase methods. Stahl et al. (1993) recently noted that additional β-carotene isomers may be present in certain human tissues, and that development of methods employing different stationary supports may provide additional information regarding the profile of carotene isomers present in serum. Trace amounts of analyte absorbing at 410 nm in areas of the chromatogram where *cis*-β-carotenes are likely to occur were occasionally noted in certain of the samples analyzed. However, like 9- and 15-*cis*-β-carotenes, these compounds were present at a level below that of the assay detection limit, if at all.

Information complementary to reversed-phase separations may also be supplied by HPLC employing calcium hydroxide. Mono-*cis* isomers of cyclic carotenes having peripherally located *cis*-bonds, such as 9-*cis*-β-carotene, characteristically have longer retention times than the *all-trans* isomer, while the mono-*cis* isomers having centrally located *cis*-bonds, such as 13- and 15-*cis*-β-carotenes, characteristically have shorter retention times than the *all-trans* compound (Zechmeister, 1962a). Thus, the unambiguous observation that 9-*cis*-β-carotene was not present in the pooled serum samples analyzed in this study is confirmation of a similar observation made previously by Stahl et al. (1992, 1993a).

In summary, geometric β-carotene isomers are undoubtedly formed during food processing (Sweeney and Marsh, 1971; Chandler and Schwartz, 1988) and may be present naturally in some edible preparations (Craft et al., 1990), meaning that the ingestion of significant quantities of these compounds in many human populations is a certainty. Little is known about the absorption and transport of individual geometric isomers in mammals, although one study on the absorption of lycopene isomers from raw and processed tomato juice (Stahl and Sies, 1992) and one study reporting on the ferret as a model for the absorption of β-carotene isomers in humans (White et al., 1993) were recently completed. Further innovative research will be required to understand the absorption and potential biological impact of geometric isomers in humans. The assay described herein provides a procedure by which the predominant geometric β-carotene isomers present in human serum can be measured in such studies.

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