which forms under appropriate conditions when the ΔF508 and normal alleles are present. The heteroduplex migrates more slowly than do homoduplex fragments and can be separated in 20 min by electrophoresis and detected with ethidium bromide staining. To reliably distinguish homozygous normal and affected individuals with this method, two electrophoresis lanes per sample must be run. One lane contains the PCR-amplified patient’s sample, and the other contains the same PCR product supplemented with an equivalent amount of PCR product from a noncarrier of the mutation (5). Alternatively, the ΔF508 mutation has been detected directly by 2-h electrophoresis followed by ethidium bromide staining (6) or autoradiography from emission of a radiolabeled primer used for PCR amplification (7). An overnight restriction enzyme digestion of PCR product followed by a 2-h electrophoresis has also been a successful method of detecting the ΔF508 mutation (8).

Electrophoretic methods will probably be most suitable for laboratories with a low volume of testing. Although these methods do not require radiolabeled probes, they do require the reagents, labor, and equipment associated with electrophoresis. Comparison of methods should take into account time associated with gel preparation, loading, and staining, as well as the number of specimens that can be evaluated, given the size of the electrophoresis apparatus. Unlike the ASO–PCR dot–blot method, which has the potential for the automation of membrane preparation and data analysis, these tasks must be done manually for electrophoresis. Additionally, possible analytical problems associated with sample overload, variation in migration, nonspecific bands, and differences in the amount of PCR product have not been ascertained for electrophoresis.

The ASO–PCR dot–blot method described will probably be most appropriate for higher-volume testing programs, such as those for newborn or population carrier screening. With this method, one can hybridize, wash, and analyze membranes containing hundreds of specimens at one time. Compared with electrophoresis, this method can provide higher sample throughput, amenability to automation, and the demonstrated low incidence of repeat analysis. Adopting the method to tolerate impure DNA samples, thereby minimizing sample preparation time, would further increase its utility for clinical testing and screening.

References

Determination of β-Carotene and Its Cis Isomers in Serum

W. Gray Rushin, George L. Catignani, and Steven J. Schwartz

All-trans-β-carotene was resolved from its cis isomers in human serum by reversed-phase "high-performance" liquid chromatography. Absorption spectra of the cis peak suggested that 13-cis-β-carotene was the predominant cis isomer. Analyses and recovery studies of fresh and stored sera eliminated the possibility that isomerization had occurred in samples during handling or storage. The average analytical recovery was 101.9% for standards of the all-trans, 9-cis, and 13-cis-β-carotene compounds in pooled serum samples. We also demonstrated that cis isomers had not formed after the blood was drawn and that cis isomers of β-carotene are present at significant concentrations in the human circulation.

Additional Keyphrases: chromatography, reversed-phase nutritional status

All-trans-β-carotene (ATBC) in fruits and vegetables has been shown to undergo cis isomerization during processing (1–7), with the 9-cis-β-carotene (9-CBC) and 13-cis-β-carotene (13-CBC) forms being the predominant isomerization products. Thus consumption of a typical diet involves ingestion of significant quantities of cis isomers. However, the metabolic fate of the isomers is unknown. Several animal-feeding studies, measuring growth or the concentration of vitamin A in tissue after ingestion of β-carotene isomers, have demonstrated that cis rotation of ATBC can substantially reduce its provitamin A activity (8–11).

Department of Food Science, North Carolina State University, Raleigh, NC 27695-7824.
1 To whom correspondence should be addressed.
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Methods developed to evaluate the concentration of ATBC in plasma and serum (12–15) have detected the 15-cis isomer in human plasma (14, 16) as well as an unidentified "cis isomer" (17). However, adequate techniques for detecting the predominant dietary isomers of ATBC, namely, 9- and 13-CBC, have not been developed. Here we report the presence and quantification of ATBC and its cis isomers in human serum.

Materials and Methods

Pooled serum samples (20 mL) received each week from the North Carolina Department of Public Health in Raleigh, NC, were stored at −20 °C until assay. Solvents were HPLC grade (Fisher Scientific, Raleigh, NC) and were degassed by filtration under reduced pressure through a 0.45-μm pore-size filter. All sensitive procedures were performed under subdued lighting.

Preparation and identification of standards. Standards for ATBC, 9-CBC, and 13-CBC were isolated and collected from canned spinach. Tissue extraction and column-packaging procedures (with Ca(OH)₂) were performed as previously described (6). Each isomer was purified on a semi-preparatory Ca(OH)₂ column, a 7.8 × 61 cm stainless-steel column (Waters Inc., Division of Millipore Corp., Milford, MA). We analyzed the standards for purity and identity by checking their ultraviolet-visible wavelength spectra with a Waters Model 590 photodiode array detector. Mass spectra were determined with a Hewlett-Packard (Palo Alto, CA) 5966B mass spectrometer performed at the GC/MS Facility, North Carolina State University, Raleigh, NC, and the 1H nuclear magnetic resonance (NMR) spectra with a Bruker (Rheinstetten, F.R.G.) 250-MHz 1H-NMR spectrometer at the Research Triangle Institute, Research Triangle Park, NC. These spectra were compared with previously published data (7, 18). NMR samples were prepared in deuterated chloroform. Standard concentrations were calculated from published absorptivity values for standards in hexane (19), with a Model 250 spectrophotometer (Gilford Instrument Laboratories, Oberlin, OH), then stored at −20 °C in hexane.

Serum sample preparation. For serum analysis, pipette 2 mL of serum and 2 mL of ethanol into a 12 × 75 mm test tube, vortex-mix for 20 s, then add 2 mL of hexane. Vortex-mix (1 min) and centrifuge (500 × g, 5 min), then remove 100 μL of the organic layer, and evaporate it under N₂. Redissolve the residue in 20 μL of anhydrous ether followed by 80 μL of methanol, and inject 80 μL of this into the HPLC system.

HPLC instrumentation and conditions. The HPLC system consisted of a Model 510 pump, a Model U6K injector, and a Model 990 photodiode array detector (Waters Inc.) equipped with an APC IV series computer (NEC Information Systems, Inc., Foxborough, MA) for spectral analysis of the isomers. To quantify the isomers, we used the more sensitive Linear UVIS-203 detector (ANSPEC, Ann Arbor, MI), with a compensating polar planimeter (Keuffel & Esser Co., Morristown, NJ) to integrate the areas of the all-trans and cis peaks. We used two 5-μm-particle Vydeac 80TP54 columns (Rainin, Woburn, MA) in series to achieve optimum resolution. The solvent system consisted of methanol:chloroform:tetrahydrofuran (87:10:3 by vol), flowing isocratically at 0.5 mL/min. Column effluent was monitored at 436 nm, and detector sensitivity was set at 0.003 absorbance units full scale.

Recovery studies. To 2-mL aliquots of various pooled serum samples, we added 200 μL of hexane (control) or 200 μL of a known concentration of each ATBC, 9-CBC, and 13-CBC standard, then extracted and analyzed as above. We also kept a duplicate control sample at room temperature in the dark for 48 h before extraction and analysis.

Fresh serum study. Blood samples drawn from two student volunteers at the North Carolina State University campus infirmary were immediately placed on ice in the dark, allowed to clot for 15 min, and centrifuged (500 × g, 10 min). We removed the serum and analyzed 2-mL aliquots immediately and after 48 h.

Statistical analyses. We used a Student’s paired t-test to determine the significant differences (P <0.05) in the serum ATBC and cis-isomer concentrations before and after various treatments.

Results and Discussion

Previous researchers (1–3, 6, 7, 20) have shown that calcium hydroxide is an effective adsorbent for resolving the cis isomers of β-carotene. With a semi-preparative Ca(OH)₂ column, we resolved each of the three major (ATBC, 13-CBC, and 9-CBC; and one minor, 15-CBC) isomers in foods, with sample loads ≤2 mg of β-carotene extracted from canned spinach. Consequently, these columns were targeted for use in developing the serum assay. However, serum constituents in both saponified and unsaponified serum extracts could not be resolved with the calcium hydroxide columns.

On the basis of previous data (4, 5, 21), we selected the Vydeac 201TP54 column as having the potential to separate the cis isomers of β-carotene, even though in previous studies, compound identification was inconsistent. Using two columns in series and a mobile phase of methanol:chloroform:tetrahydrofuran (87:10:3 by vol), we resolved the cis isomers in serum from ATBC.

We identified ATBC in serum by co-chromatography with an authentic standard (Figure 1a and b) and by comparing visible absorption spectra. We also separated ATBC and the cis isomers; however, the individual cis isomers were not resolved. The standards of 9-CBC and 13-CBC co-eluted with the cis-isomer peak identified in Figure 1c and d. The spectral scan of this peak in several pooled samples of serum matched the 13-CBC standard, but in others, the "cis peak" in the 340-nm region was intermediate between 9-CBC and 13-CBC. This suggests that the cis-isomer peak was predominantly 13-CBC, but that smaller amounts of 9-CBC were also present. However, without resolution between the individual isomers, we can only speculate about the relative percentages of the isomers. In fact, small quantities of other cis isomers, e.g., the 15 cis-isomer or the di-cis forms, could be present. The spectral scan of the cis-isomer peak revealed that this peak is composed solely of forms of β-carotene and is not contaminated with other carotenoids or noncarotenoids.

The assay showed that in pooled human serum cis isomers were present at concentrations ranging from 20% to 45% of the all-trans form. ATBC recovery results appear in Table 1. Although we are aware that added standards do not equilibrate with lipoprotein-bound carotenoids (14), recovery studies eliminate the possibility that extraction, sample preparation, column effects, or the organic solvents of the mobile phase induce isomerization. With ATBC, the quantity of the cis isomers did not change significantly from the control sample (75 μg/L) to the recovery sample (72 μg/L). Additionally, 99.7% of the standard was recov-
The 9-CBC and 13-CBC recovery results (Table 1) also indicated that no isomerization took place during sample handling. Furthermore, the 48-h sample results (Table 2) showed that room-temperature conditions did not lead to further isomerization and suggested that prior handling conditions, i.e., delivery to or from the Department of Public Health, were not responsible for the presence of the cis isomers.

Table 2 also shows the assay results for freshly drawn serum samples. Evaporation caused the concentration of ATBC and its cis isomers to increase slightly over 48 h. Had ATBC isomerized to 9-CBC or 13-CBC over 48 h, the ATBC/CBC ratio would have decreased significantly. However, the ratio for both samples remained virtually unchanged; thus, the isomers were not formed after the blood was drawn.

Recovery results and studies of fresh serum demonstrate that significant quantities of β-carotene cis isomers are present in human blood. Our data provide the relative amounts of ATBC and its cis isomers in serum. The serum assay also provides a possible method to establish the relationship between dietary intake and circulating concentrations of ATBC and its cis isomers.

Using 10 fresh and processed fruits and vegetables, Chandler and Schwartz (6) determined the relative values of ATBC, 9-CBC, and 13-CBC for many foods rich in β-carotene. Panalaks and Murray (2) and Sweeney and Marsh (1, 3) provided similar data for a variety of fresh, frozen, and canned fruits and vegetables, both cooked and uncooked. The mean trans/cis ratio in fruits and vegetables was 3.05 from Chandler and Schwartz (6), 2.94 from Panalaks and Murray (2), and 3.48 from Sweeney and Marsh (1, 3). The ratios for pooled and fresh serum samples were 3.01 and 3.15, respectively. The similarity in these ratios suggests that previously reported preferential absorption of ingested ATBC relative to the cis isomers was substantially overestimated (22).

Jensen et al. (22) conducted a human feeding study to determine the effects of ingesting cis- and trans-β-carotene isomers on serum concentrations. In their work, the source of the cis-isomers was β-carotene extracted from Dunaliella salina alga, which reportedly contains approximately equal amounts of ATBC and 9-CBC (22). Both the fasting serum concentrations and the post-diet concentrations revealed trans/cis ratios of ~10.3. From these data, they suggested that the intact trans form is preferentially absorbed over intact 9-CBC. Their data and conclusions disagree with the results presented here. Reviewing the methodology, Jensen et al. (22) used suggests possible reasons for this discrepancy: injecting a hexane extract directly into a methanol/acetonitrile mobile phase could pose solubility problems. Furthermore, no recovery studies were conducted to validate the extraction, handling, and storage procedures, nor were authentic standards used to validate the identity of the cis isomers, assay accuracy, and thus their ratios.

Possibly, carotene consumption from an algal source alters the absorption (or transport) process by favoring uptake of the trans-isomer or inhibiting uptake of the cis-isomers. However, Ben Amotz et al. (23) recently reported that the ratio of 9-CBC to ATBC in liver of algae-fed chicks is higher than that in the algae itself, which sug-

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Table 2. Stability of Isomers in Three Pooled and Two Fresh Serum Samples Assayed Initially and after 48-h Incubation at Room Temperature

<table>
<thead>
<tr>
<th>Sample</th>
<th>n</th>
<th>All-trans</th>
<th>Cis Isomers</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pooled</td>
<td></td>
<td>All-trans</td>
<td>Cis Isomers</td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>4</td>
<td>199 (15)</td>
<td>73 (5)</td>
<td>2.72</td>
</tr>
<tr>
<td>+ 48 h</td>
<td>3</td>
<td>184 (12)</td>
<td>73 (2)</td>
<td>2.52</td>
</tr>
<tr>
<td>Initial</td>
<td>3</td>
<td>231 (67)</td>
<td>67 (10)</td>
<td>3.45</td>
</tr>
<tr>
<td>+ 48 h</td>
<td>3</td>
<td>230 (67)</td>
<td>67 (10)</td>
<td>3.43</td>
</tr>
<tr>
<td>Fresh</td>
<td>1</td>
<td>164</td>
<td>70</td>
<td>2.34</td>
</tr>
<tr>
<td>+ 48 h</td>
<td>1</td>
<td>176</td>
<td>76</td>
<td>2.32</td>
</tr>
</tbody>
</table>
gests a selectivity opposite that reported by Jensen et al. (22).

The decrease in vitamin A activity resulting from the cis isomerization of ATBC (3) suggests that most, if not all, published data concerning the vitamin A nutritive value in fruits and vegetables are largely overestimated. Furthermore, whether cis isomerization affects other chemical or biological properties of ATBC—e.g., its ability to serve as an anti-oxidant (24) or singlet oxygen quencher (25), or its possible roles in health maintenance and disease prevention—is unknown.

This study demonstrates the presence of cis isomers of β-carotene in human serum and provides a method for their quantification. In addition, the data suggest that the cis isomers, which are naturally present or formed by the processing of fruits and vegetables, are absorbed and circulated intact in human plasma and that these isomers might not be differentially absorbed from dietary sources relative to ATBC. The absorption, transport, and biological role of these isomers should provide interesting areas for further research.

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