Carotenoids in the Human Macula and Whole Retina

Garry J. Handelman, Edward A. Dratz, Collin Reay and Frederik J. G. M. van Kuijk

The carotenoid pigments in the whole human retina and in the macular region were measured quantitatively by high pressure liquid chromatography (HPLC). Approximately a five-fold larger amount of carotenoids was found in the human macula (35–120 ng) than in previously reported work. The dominant carotenoids in the whole retina are lutein and zeaxanthin. Zeaxanthin is concentrated in the macular region, whereas lutein is dispersed throughout the entire retina. Contrary to prior reports, substantial quantities of both carotenoids are present in the infant retina. Increasing variability is observed in carotenoid levels between individuals with advancing age, and some older individuals show very high whole retina carotenoid levels. These quantitative studies were made possible by synthesis of a new, stable carotenoid internal standard. Carotenoids have been proposed to be potent antioxidants, protecting membrane lipids from toxic peroxidation reactions. The method presented in this study will facilitate quantitative investigations of the association between carotenoid levels and health and disease of the retina.

The first effort at chemical identification of the human macular pigment was reported by Wald, who tentatively identified the pigment as a carotenoid or carotenoids, belonging to the leaf xanthophyll family. Wald's findings were based on the absorption spectrum and organic solvent partition characteristics of the pigment. Recently, Bone et al have published evidence that the pigment is a mixture of two carotenoids, lutein and zeaxanthin. They estimated that about 10 ng of total carotenoid could be extracted per macula.

Data from psychophysical measurements indicate a large variation in the amount of pigment between different adults. Pease and Adams recently found a six-fold variation in macular pigment among different adult subjects and Werner et al found about an eight-fold variation. Wald and Bone and Sparrock noted that there was no detectable pigment in some apparently normal adult subjects, and there are reports that no macular pigment is present in the infant. Pigment was also measured in histological sections of monkey retinas by microspectrophotometry, and the absorption spectra were consistent with the presence of carotenoids. The peak macular pigment absorbance measured by this technique varied about three-fold between different monkey retinas.

The biological role of this macular pigment is still uncertain. Various roles have been proposed, including limiting chromatic aberration at the fovea by filtering out blue light, quenching of singlet oxygen or free radicals produced in the retina, and protecting the macula from the phototoxicity of blue light.

The present study is based on a method for separation and quantitative determination of the carotenoid pigments that is linear and shows high recovery. The method was applied to analysis of the whole retina as well as the macular region. Between 20 and 250 ng of carotenoid/retina was found in different individuals.

Materials and Methods

Reagents and Equipment

HPLC grade acetonitrile, methanol and isopropanol (Fisher) were used for HPLC mobile phases. Hexanes were redistilled, reagent grade; ethanol was 200 proof, USP grade, redistilled (Publicker, Linfield, PA); and H2O was HPLC grade (Burdick and Jackson, Muskegon, MI). /3-apo-8'-carotenal and O-ethylhydroxylamine were from Fluka, Ronkonkoma, NY. O-methyl-hydroxylamine was from Aldrich. Lutein and zeaxanthin were kindly donated by Hoffman-La Roche (Nutley, NJ) and N. I. Krinsky (Tufts University School of Medicine, Boston, MA). /3-carotene and all-trans retinal were from Sigma (St. Louis, MO).

Analyses were carried out with an Altex gradient HPLC system, with Model 110A pumps, Model 453...
controller, and Rheodyne 725 injector with a 20 µl loop. Peak detection at 450 nm was done using a Perkin-Elmer LC-95 HPLC detector equipped with a 1.6 cm path length 18 µl flow cell. A Spectra-Physics Minigrator was used to monitor retention times. Peaks were recorded on a Hewlett-Packard 7130 chart recorder.

Carotenoid analyses were done with a modification of the method of Krinsky and Welankiwar. Routine analyses were carried out by isocratic elution in 85/15 acetonitrile/methanol at 1.0 ml/min. The chromatographic column was an Alltech Econosphere C18 with 5 µm particle size and 10% carbon load. Column dimensions were 25 cm × 0.46 cm, with C18, 5 µm, 1 cm × 0.46 cm integral cartridge pre-column. For determinations of nonpolar carotenoids, such as β-carotene, a step-gradient to 20% isopropanol in the acetonitrile/methanol mobile phase was used after the more polar carotenoids were eluted.

Internal Standard

The structure of a new, highly stable internal standard that was prepared for this work is shown in Figure 1. The standard is the O-ethylhydroxylamine derivative of β-apo-8'-carotenal (called carotenal-ethyl-oxime) and was made by a procedure adapted from the method of van Kuijk et al. Two milliliters of a 1 mg/ml solution of apo-carotenal (in methanol) were mixed with 200 µl of a solution of 0.1 M ethyl-hydroxylamine (in 0.1 M PIPES buffer, adjusted to pH 4.7) and incubated 12 hr in the dark at room temperature. The derivative was purified by HPLC, using the isocratic mobile phase conditions described above. The HPLC mobile phase was evaporated, and the carotenal-ethyl-oxime redissolved in hexanes for storage. The optical density of the stock solution of the carotenal-ethyl-oxime was determined at 450 nm and a dilution in hexanes prepared with 0.01 absorbance from which 1 ml was used in each analysis. If columns with higher carbon loading are used, the retention time of the internal standard may be excessive and other internal standards synthesized from shorter precursors (e.g., β-apo-12'-carotenal from Hoffmann-LaRoche) should be substituted.

Source and Preparation of Tissues

Specimens were obtained from donor eyes provided by the Lions Eye Bank, Pacific Presbyterian Medical Center, San Francisco, CA. The corneas had been removed from most of the donor eyes. Eyes were either dissected at the Eye Bank to remove the vitreous humor and the posterior poles frozen at -70°C, or intact globes were frozen. The eyes or posterior poles were usually frozen within 48 hr of death. Storage at -70°C was for a maximum of 1 year before analysis. Paired specimens from individual donors were frozen and thawed at the same time. Retinas were analyzed separately from both eyes from a number of donors. For several donor eye pairs, the macular region from one eye was analyzed and compared to the whole retina from the fellow eye. Only one eye was available from some donors, and the whole retina was analyzed in these cases.

Precise anatomical collection of the macular region from thawed frozen eyes was not possible unless the macular regions were obtained from specimens from which the vitreous was removed before freezing. If a frozen whole globe was processed, it was used to obtain the complete retina. The dissection procedure was directed at obtaining the region of the retina between the superior and inferior temporal vascular arcades, as this region is easily visualized and the macula is fully contained within these boundaries. A sharp 8 mm trephine was used to punch out the approximately 5 mm diameter macula with some surrounding tissue, and this region was then lifted off with a forceps. All of the macula was removed with the trephined section, along with about 5% of the peripheral retina surrounding the macula.

Extraction of Tissues

Retina samples were placed in pre-tared glass homogenizer sleeves. Buffer (10 mM HEPES, pH 7.4, 0.1 M NaCl, 1 mM Na₂EDTA) was added to bring the weight of sample plus buffer to 0.5 g, and 0.5 ml ethanol containing 50 µg/ml butylated hydroxy toluene (BHT) was added. The sample was homogenized for 30 seconds with a Teflon pestle. The homogenate was transferred to an 8 ml vial with a Pasteur pipette, and 1 ml of the internal standard solution in hexanes was added, followed by 3 ml hexanes. The vial was
sealed with a Teflon-lined cap, mixed on a vortex mixer for 120 seconds, and centrifuged at 800 g for 30 seconds. The upper phase was transferred to a clean vial and evaporated under nitrogen at 40°C. The dry film was dissolved in 60 μl of methanol, which is effective in fully solubilizing the extract for HPLC analysis. A 20 μl portion was analyzed promptly by HPLC with detection at 450 nm. All manipulations were carried out under incandescent bulbs shielded with orange filters.

Calibration

The standard solution of zeaxanthin for calibration was made in acetonitrile from solid supplied by Hoffmann-La Roche, and its concentration was determined spectrophotometrically from the molar extinction of 134,000 1/mole-cm at 452 nm.13 Lutein from Hoffmann-La Roche was purified by HPLC, and its concentration in acetonitrile was determined from the molar extinction at 448 nm of 134,000 1/mole-cm.13 Standard solutions were divided into small portions, stored in the dark at -20°C, and were shown to be stable for up to 30 days under those conditions. On the day of analysis, a fresh portion of each standard solution was diluted in acetonitrile to give a concentration of 1.0 μg/ml of each carotenoid in the working stock solution. Fifty microliters of the stock solution was added to 0.5 ml EtOH and 0.5 ml H2O and analyzed in parallel with the tissue extracts. Peak height ratios between the known amounts of standards and internal standard were measured to calibrate the method for unknown quantitation. The absorption maximum of the native peak 1 collected from the HPLC was 381 nm with a weak tail that reached beyond 450 nm, the same as all-trans-retinal.14 None of the carotenoid peaks changed their retention time. Peak 1 in underivatized and derivatized extracts had retention times identical with native and identically derivatized all-trans-retinal, respectively. The absorption maximum of the native peak 1 collected from the HPLC was 381 nm with a weak tail that reached beyond 450 nm, the same as all-trans-retinal.14 None of the carotenoid peaks changed their mobility in the presence of O-methyl hydroxylamine.

After the elution of the internal standard, no peaks of significant amplitude were detected which could interfere with lutein and zeaxanthin on chromatographic analysis of subsequent samples. Only traces of nonpolar carotenoids, such as β-carotene were detected (see below). Unidentified satellite peaks are present surrounding the main carotenoid peaks (Fig. 2A).

Table 1 shows the comparison of carotenoid content from the left and right retinas from eight subjects. The agreement between the amounts of lutein and zeaxanthin in the right and left retina for each donor is quite good. However, some difficulties in quantitative dissection of the retina were noted and improvements in quantitative collection of the retina may show even closer agreement.

When intact eyeballs were frozen, the specimens could usually be processed to collect the whole retina after thawing. In about 75% of the cases, most of the retina could be collected from a thawed eyeball, with little vitreous or choroid contamination. About 25%
Fig. 2. HPLC chromatographs monitored at 450 nm. (A) Whole human retina. (B) Macular region of the fellow eye that provided the retina in (A). (C) Reference standard lutein (2), zeaxanthin (3) and carotenal-ethyl-oxime internal standard (IS). The identity of the peak labeled 1 is discussed in the text.

Table 1. Lutein and zeaxanthin content in whole retinas from donors where both eyes were analyzed (values in nanograms carotenoid per retina)

<table>
<thead>
<tr>
<th>Donor no.</th>
<th>Age</th>
<th>Lutein Left</th>
<th>Lutein Right</th>
<th>Zeaxanthin Left</th>
<th>Zeaxanthin Right</th>
</tr>
</thead>
<tbody>
<tr>
<td>1)</td>
<td>1 wk.</td>
<td>24.8</td>
<td>25.4</td>
<td>10.2</td>
<td>10.1</td>
</tr>
<tr>
<td>2)</td>
<td>2 mo.</td>
<td>23.4</td>
<td>21.3</td>
<td>10.3</td>
<td>10.0</td>
</tr>
<tr>
<td>3)</td>
<td>10 yrs.</td>
<td>15.0</td>
<td>16.0</td>
<td>8.3</td>
<td>8.6</td>
</tr>
<tr>
<td>4)</td>
<td>15 yrs.</td>
<td>24.7</td>
<td>18.0</td>
<td>18.3</td>
<td>13.4</td>
</tr>
<tr>
<td>5)</td>
<td>32 yrs.</td>
<td>45.9</td>
<td>50.2</td>
<td>18.8</td>
<td>36.6</td>
</tr>
<tr>
<td>6)</td>
<td>56 yrs.</td>
<td>68.6</td>
<td>58.4</td>
<td>35.0</td>
<td>30.0</td>
</tr>
<tr>
<td>7)</td>
<td>57 yrs.</td>
<td>62.2</td>
<td>49.3</td>
<td>42.1</td>
<td>30.6</td>
</tr>
<tr>
<td>8)</td>
<td>81 yrs.</td>
<td>59.6</td>
<td>43.6</td>
<td>26.0</td>
<td>17.8</td>
</tr>
</tbody>
</table>

of frozen eyebank specimens were suitable for proper collection of the macula. Both procedures, collection of the retina or the macular region, were somewhat approximate techniques from eyebank samples in our hands. Some retinal tissue always adheres to the eyeball. The trephine tends to traumatize the tissue so that it is difficult to collect the complete macula. With more extensive experience, it may be possible to establish conditions to obtain maculas in high yield from eyebank specimens.

Figure 3 summarizes the amounts of lutein and zeaxanthin in whole retinas, from 16 subjects over a broad age range. If two retinas were analyzed per subject, the average result is given. There is substantial carotenoid in the infant retinas analyzed. The highest values we observed were found in adulthood and old age, reaching the values of 94 ng and 186 ng of lutein in the whole retina of two elderly subjects. However, for some adult retinas, the lutein value is near the value observed for the very young retinas.

Fig. 3. Carotenoid content of human retinas from donors of different ages. The closed circle is the number of nanograms of lutein per retina and the closed triangle is the number of nanograms of zeaxanthin per retina. Thirteen of the samples are averages from the paired retinas shown in Table 1, and three samples are from single retinas.
the two carotenoid pigments seem present in roughly
anthin than lutein in the whole retina in 15 of the 16
lutein). For zeaxanthin, the macular
was analyzed from one eye and the macular region
from the fellow eye. The amount of lutein in the
samples analyzed (zeaxanthin averaged 44% of the
macular region is usually about 30% to 60% of the
whole retina value. For zeaxanthin, the macular
value is 70-110% of the whole retinal value. Further,
the two carotenoid pigments seem present in roughly
equal amounts in the macular region as was also re-
ported by Bone et al.2

Analysis of Retina and Macula For β-Carotene and
Other Nonpolar Carotenoids

A step gradient to 20% isopropanol in the acetoni-
trilte/methanol mobile phase after the internal stan-
dard has eluted accomplishes the elution of nonpolar
carotenoids as sharp peaks, including lycopene, α-
carotene, and β-carotene if they are present. Three
macular regions were analyzed for nonpolar carot-
enoids, and no significant β-carotene or other non-
polar carotenoid could be detected even though typical
amounts of lutein and zeaxanthin were found.
Two whole retinas were also analyzed for nonpolar
carotenoids. β-Carotene equivalent to 1% of the lute-
in was found in one and to 3% in the other whole
retina. Traces of other nonpolar carotenoids could
also be seen.

Discussion

The data presented support the qualitative obser-
vation of Bone et al2 that lutein and zeaxanthin are
the dominant carotenoids in both retina and macula.
We hypothesize that the very small amounts of non-
polar carotenoids found were contributed by blood
contamination of the autopsy retinas and that essen-
tially no β-carotene is present in the human retina.
The present method is designed to be quantitative,
and we find much larger amounts of carotenoids than
published by Bone et al.2 The method used by Bone
et al2 was designed for identification, not quantita-
tion. Bone et al have previously reported in an ab-
tract15 amounts of macular carotenoid in quantita-
tive agreement with the finding reported here.

The data presented indicate that substantial carot-
enoid is found in the retina shortly after birth. The
data also suggest that the pigment accumulates in the
whole retina with age to much higher levels in some individuals, but not in others (Fig. 3), although there
are not enough samples for statistical evaluation.
This suggestion is consistent with psychophysical
measurements1,3-5 that found the density of the mac-
ular pigment in different normal subjects ranged
widely from undetectable to 1.2 absorbance units.
While variation within an age group has been re-
ported by several investigators, it has also been re-
ported that the macular pigment density does not
change systematically with age, as measured psycho-
physically.3-5 Bone et al in a recent abstract also find a
lack of systematic accumulation with age.16 It may be
that the pigment density remains fairly constant in
the macula but that it accumulates, in some individ-
uals, in the peripheral retina. This question should be
addressed by analysis of a larger number of maculae
and retinas over a broad age range.

Retinal carotenoids are of interest since their
dietary intake may confer antioxidant protection.
There are indications that human age-related macu-
lar degeneration may be associated with deficiencies
in antioxidant protection.9,17 Carotenoids are the
most active protective agents known against highly
reactive singlet oxygen,18 and singlet oxygen-induced
lipid peroxidation has been proposed to be a media-
ton of light damage in the retina.9,10 New gas chroma-
tography-mass spectrometry methods19,20 have pro-
vided evidence that lipid peroxidation products accu-
mulate in rat and dog retinas degenerating due to
vitamin E deficiency.21 Some of the peroxidation
products present are highly cytotoxic.22 Recently, it

<table>
<thead>
<tr>
<th>Donor no.</th>
<th>Age (yrs.)</th>
<th>Whole retina</th>
<th>Macular region</th>
<th>Macula/retina†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) 38</td>
<td>78.9</td>
<td>48.2</td>
<td></td>
<td>39.9</td>
</tr>
<tr>
<td>2) 65</td>
<td>15.8</td>
<td>17.2</td>
<td></td>
<td>15.7</td>
</tr>
<tr>
<td>3) 72</td>
<td>29.0</td>
<td>17.7</td>
<td></td>
<td>18.3</td>
</tr>
<tr>
<td>4) 79</td>
<td>93.8</td>
<td>49.4</td>
<td></td>
<td>39.9</td>
</tr>
<tr>
<td>5) 81</td>
<td>182.0</td>
<td>83.0</td>
<td></td>
<td>68.3</td>
</tr>
</tbody>
</table>

* Zea. = zeaxanthin.
† These columns show the ratio of lutein and zeaxanthin in the macular region.

Figure 3 and Table 1 show that there is less zeax-
thin than lutein in the whole retina in 15 of the 16
samples analyzed (zeaxanthin averaged 44% of the
lutein).
has been proposed by Krinsky and Deneke\textsuperscript{23} and by Burton and Ingold\textsuperscript{24} that carotenoids may also function as highly effective free radical trap antioxidants at the low oxygen tension found in tissues. Carotenoid-deficient monkeys were reported to show pigmentary changes in the fundus.\textsuperscript{25} However, controls for the specific effects of dietary carotenoid supplementation to the deficient diet were not carried out.

If carotenoid pigments are serving a protective function, it would be of interest to compare the concentration of the pigment in humans with degenerative retinal disease, such as age-related macular degeneration, with age-matched controls. Studies of possible association between carotenoid levels and human health are of additional interest because both psychophysical and biochemical data suggest substantial variation between different individuals. Experiments could be undertaken in primates to alter retinal carotenoid levels by dietary means and to study possible changes in resistance to degeneration resulting from these manipulations.

**Key words:** carotenoids, lutein, zeaxanthin, human retina, macula, HPLC, lipid peroxides, age related macular degeneration

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**References**