HPLC Analysis of Vitamin E by Conversion to α-Tocopheryl Acetate in Samples Containing Canthaxanthin or Other Coeluting Compounds

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Canthaxanthin, a nonprovitamin A carotenoid and food coloring agent, and α-tocopherol (vitamin E) often coexist in foods and tissues. As both canthaxanthin and α-tocopherol are thought to be lipid-soluble antioxidants, it is sometimes necessary and often desirable to determine the concentrations of both compounds in the same sample extract. We have previously reported that canthaxanthin beadlets, capsules, and tablets, primarily used for food-coloring purposes, contained *cis*- and *trans*-canthaxanthin and two other carotenoid-like compounds. One of these compounds was found to coelute with α-tocopherol by conventional HPLC methods of analysis of vitamin E. A reversed-phase HPLC method was therefore devised to quantitate low levels of α-tocopherol in the presence of canthaxanthin and its accompanying chromophores by first converting α-tocopherol to its acetate derivative. Acetylation resulted in a more hydrophobic analyte and a correspondingly increased retention time. By this method it was found that chicks fed semipurified diets supplemented with canthaxanthin beadlets had a significantly higher mean hepatic α-tocopherol content, as well as a significantly lower mean hepatic retinol content, than chicks fed diets supplemented with carotenoid-free placebo beadlets.

α-Tocopherol (vitamin E) is a lipid-soluble compound whose principal function is to protect polyunsaturated lipids in biological systems from oxidative degradation. α-Tocopherol can be measured by a variety of relatively nonspecific techniques, including colorimetry, spectrophotometry, and spectrofluorometry, as well as by more specific chromatographic methods. The latter include paper chromatography, thin-layer chromatography, high-performance liquid chromatography (HPLC), and gas–liquid chromatography (GLC) (Desai and Machlin, 1985). HPLC is generally the method of choice for the analysis of α-tocopherol in government and industrial laboratories (Desai and Machlin, 1985). Many different HPLC methods for α-tocopherol have been reported, using either reversed-phase (De Leenheer et al., 1978) or normal-phase (Tangney et al., 1979) HPLC, as well as HPLC with fluorometric detection (Desai and Machlin, 1985; Hatam and Kayden, 1979).

β-Carotene and canthaxanthin are naturally occurring carotenoids that are approved for use as food colorants. These carotenoids, like α-tocopherol, are also believed to play a role as lipid-soluble antioxidants. β-Carotene has been shown to have antioxidant properties in a variety of experimental systems (Burton and Ingold, 1984; Foote and Denny, 1968; Krinsky and Deneke, 1982; Terao et al., 1980; Krasnovsky and Paramonova, 1983; Blakely and Knight, 1986). Likewise, the nonprovitamin A carotenoid canthaxanthin has been shown to quench singlet oxygen and other radical species in vitro (Krinsky and Deneke, 1982; Mathews-Roth, 1986). As carotenoids and vitamin E may have similar antioxidative functions and coexist in food or tissue extracts, it is sometimes necessary and often desirable to measure α-tocopherol and the carotenoid(s) of interest in the same sample extract. We have previously measured β-carotene, retinol, and α-tocopherol in tissues by HPLC and reported that dietary β-carotene significantly increased the concentration of α-tocopherol and retinol in chick liver and subcellular fractions (Mayne and Parker, 1986). However, an attempt to determine the influence of dietary canthaxanthin on tissue retinol and α-tocopherol levels in chicks showed that carotenoid-like compounds associated with the dietary canthaxanthin beadlet preparation were absorbed by chicks (Mayne and Parker, 1988) and coeluted with α-tocopherol by both reversed- and normal-phase HPLC methods. While pure *all-trans*-canthaxanthin did not interfere with the conventional HPLC analysis of α-tocopherol, the canthaxanthin preparations that we examined (canthaxanthin beadlets, capsules, and tablets) all contained substantial amounts of these related, interfering chromophores (Mayne and Parker, 1988). Therefore, a method was devised to measure low levels of α-tocopherol in the presence of substantial amounts of canthaxanthin and its accompanying chromophores and applied to analysis of chick hepatic tissue extracts. This report describes the method and reports on the application of this method in the determination of the influence of dietary canthaxanthin on α-tocopherol and retinol concentrations in chick liver.

MATERIALS AND METHODS

Apparatus. The HPLC system employed consisted of a Beckman 421 controller, Beckman 110A pump, a 4.6 × 250 mm column of 5-μm Ultrasphere ODS (Beckman Instruments, Altex Division, San Ramon, CA), and a Hitachi 100-10 UV/vis variable-wavelength detector. Retinol was determined at 326 nm in a mobile phase of methanol–water (95:5) at 2.3 mL/min (Bieri et al., 1979). α-Tocopherol acetate was determined at 210 nm in this same mobile phase. Eluting peaks were plotted and quantified by a Hewlett-Packard 3390A plotter-integrator, used with full-scale voltage deflection at 63 μV. Peaks were identified by comparison of retention times with those of standards.

Reagents. HPLC-grade solvents were used for all HPLC analyses. Retinol, α-tocopherol, and α-tocopheryl acetate were purchased from Sigma Chemical Co., St. Louis, MO. α-Tocopherol-free canthaxanthin beadlets (Roxanthin Red 10%; 10% carotenoid by weight) and canthaxanthin beadlets containing α-tocopherol as an antioxidant (dry canthaxanthin 10% SD, also 10% carotenoid by weight) were provided by Hoffman-La Roche, Inc. 4-(Dimethylamino)pyridine was purchased from Aldrich Chemical Co., Milwaukee, WI. All other chemicals were reagent grade or better.

Procedures. Extracts of canthaxanthin beadlets and saponified extracts of chick livers from placebo- and can-

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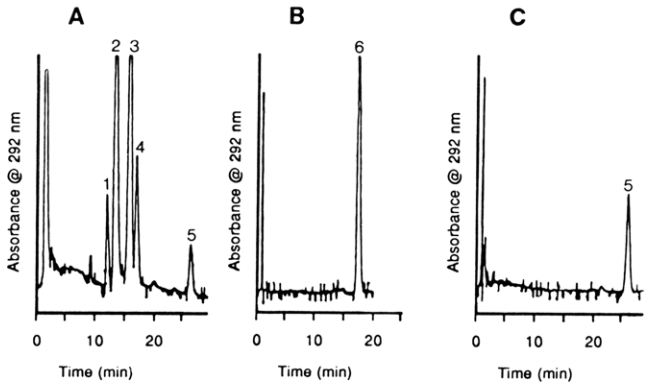

Figure 1. HPLC chromatograms: (A) acetylation products of a canthaxanthin beadlet extract containing α-tocopherol, showing *trans*- and *cis*-canthaxanthin (peaks 2 and 3), unknown carotenoid-like compounds associated with canthaxanthin beadlets (peaks 1 and 4), and α-tocopheryl acetate (peak 5); (B) α-tocopherol (peak 6), showing similar retention time to peak 4; (C) α-tocopheryl acetate (peak 5).

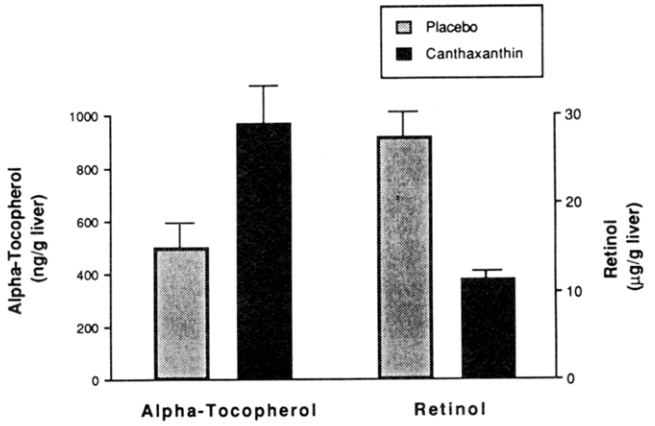

Figure 2. Effect of dietary canthaxanthin on hepatic α-tocopherol (E) and hepatic total retinol (A) concentrations in E/selenium-depleted chicks. Values are mean ± SEM (n = 12 per group for E analyses; n = 20 per group for A analyses). Dietary canthaxanthin significantly increased the mean hepatic E level ($p < 0.005$) and significantly decreased the mean hepatic A level ($p < 0.001$).

thaxanthin-fed chicks were prepared as previously described (Mayne and Parker, this volume). Liver samples were obtained from white leghorn chicks hatched from breeder hens maintained by the Department of Poultry Science, Cornell University. Chicks were fed semipurified (carotenoid-free) diets containing other placebo beadlets or 10% canthaxanthin beadlets (5.0 g of beadlets/kg diet) for 5 weeks. The diets used in this study were low in α-tocopherol and selenium so that α-tocopherol- and selenium-depleted chicks would be obtained for use in a separate study of antioxidant activity of canthaxanthin.

The described acetylation procedure uses (dimethylamino)pyridine as an acetylation catalyst (Hölfe et al., 1978) and is appropriate for sterically hindered alcohols, such as α-tocopherol. Extracts were transferred to 1 dram glass screw-cap vials with Teflon-lined caps, taken to dryness under a stream of nitrogen, and redissolved in 1 mL of pyridine. A 5-μL portion of acetic anhydride, a catalytic amount of 4-(dimethylamino)pyridine (5 mM final concentration), and Teflon stir bars were added to each vial. Vials were flushed with nitrogen, sealed, and stirred at room temperature in the dark for 48 h. Saturated sodium bicarbonate in water (200 μL) was then added to basify the solution. Samples were extracted twice with 2 mL of hexane, dried down under nitrogen, and redissolved in 95% ethanol for HPLC analysis.

RESULTS AND DISCUSSION

Analysis of α-tocopherol in extracts of stabilized canthaxanthin beadlets containing α-tocopherol showed that a carotenoid-like compound (peak 4) associated with these preparations (Mayne and Parker, this volume) eluted with a retention time indistinguishable from that of α-tocopherol (Figure 1A,B). Resolution of α-tocopherol from the four carotenoid compounds was necessary since the high concentration of the carotenoids relative to α-tocopherol in beadlet extracts and tissue samples from canthaxanthin beadlet-fed animals resulted in substantial absorption of the carotenoids at 292 nm, the absorption maximum of α-tocopherol. Numerous unsuccessful attempts at HPLC resolution were made, including (a) reversed-phase isocratic or gradient elution schemes using various methanol–water mixtures, (b) normal-phase isocratic or gradient elution using hexane–2-propanol mixtures, and (c) reversed-phase fluorometric HPLC with excitation at 291 nm and emission at 330 nm. Destruction of the interfering compounds by the sulfuric acid oxidation method of Fox and Mueller (1950) proved unacceptable due to low and variable recovery of α-tocopherol in our samples.

Derivatization of α-tocopherol in sample extracts by acetylation, followed by conventional reversed-phase HPLC analysis of α-tocopherol acetate, proved to be an acceptable alternative method of α-tocopherol quantitation. Acetylation was chosen as pure α-tocopheryl acetate is commercially available for use as a standard and is well resolved from peaks 1–4 (Figure 1A). A rigorous acetylation procedure was required as the saponified liver extracts contained substantial amounts of retinol and cholesterol, which are more easily acetylated than α-tocopherol due to steric hindrance of the free hydroxyl on the chroman head group of α-tocopherol. Recovery studies using (i) α-tocopherol standards, (ii) placebo (control) livers previously analyzed for α-tocopherol by conventional reversed-phase HPLC, and (iii) extracts of canthaxanthin beadlets (Roxanthin Red 10, which contain no α-tocopherol) to which known amounts of pure α-tocopherol were added (0.5–1.0 μg) showed that the acetylation of α-tocopherol was essentially complete with the described procedure (96 ± 6% molar recovery) and did not affect retention times of canthaxanthin or its accompanying chromophores. HPLC elution of acetylated samples was monitored at 210 nm to minimize absorbance of canthaxanthin and the unknown compounds while favoring that of α-tocopheryl acetate (the extinction coefficient of α-tocopheryl acetate in ethanol is approximately 9-fold greater at 210 nm than at 293 nm).

The influence of dietary canthaxanthin on hepatic α-tocopherol content was examined by the method described above. Dietary canthaxanthin resulted in a doubling of the mean hepatic α-tocopherol concentration relative to control (placebo-fed) chicks (Figure 2). This significant increase ($p < 0.005$, Student's t-test) is consistent with our previous reports that dietary β-carotene significantly increased α-tocopherol content in livers from chicks (Mayne and Parker, 1986) and rats (Taylor and Parker, 1985) fed diets adequate in α-tocopherol. The mechanism responsible for this increase is not clear but may involve a sparing effect resulting from the quenching of active species of oxygen by the carotenoid before their interaction with α-tocopherol, as we have previously suggested (Mayne and Parker, 1986). Regardless of mechanism, such effects of dietary β-carotene and canthaxanthin on tissue α-tocopherol must be considered a potential confounding factor in studies of biological effects of dietary carotenoids and

emphasize the need for an accurate means of quantitating canthaxanthin and α-tocopherol concentrations in the same sample.

Dietary canthaxanthin significantly ($p < 0.001$, Student's t-test) decreased the mean hepatic total retinol level 2.4-fold relative to chicks fed placebo beadlets (Figure 2). This decrease, representing a substantial change in vitamin A status, occurred despite a dietary vitamin A level (4500 IU/kg diet) which is adequate for growing chicks fed purified diets (Scott et al., 1969). Similarly, Bendich and Shapiro (1986) reported a 2.5-fold decrease in the mean retinol content of livers from rats fed supplemental canthaxanthin beadlets. The mechanism of this reduction in retinol is unknown but may involve interference by canthaxanthin or a metabolite (possibly 4-keto- or 4-hydroxyretinol) in the absorption or transport of retinol. The effect of dietary canthaxanthin on retinol status warrants further attention, as humans chronically consuming large amounts of canthaxanthin (120 mg/day) for the purpose of skin pigmentation or for the treatment of photosensitivity disorders (Gupta et al., 1985) may potentially be at increased risk for vitamin A deficiency.

The acetylation method described above is applicable in situations involving interference by canthaxanthin or when it is necessary to alter the HPLC retention time of α-tocopherol relative to other cochromatographing compounds. Under certain conditions, the analysis of other derivatives such as α-tocopheryl succinate may be preferable. However, derivatization is not specific for α-tocopherol, and the utility of any derivatization method will in part depend on the nature of the interfering compounds. In this situation, which involved interference by carotenoid compounds associated with canthaxanthin preparations, the interfering compounds apparently did not contain free hydroxyl groups available for acetylation as evidenced by the unaltered retention times of these compounds following derivatization.

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Registry No. Vitamin E, 1406-18-4; canthaxanthin, 514-78-3; α-tocopheryl acetate, 58-95-7; retinol, 68-26-8.

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