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Retinol, α-Tocopherol, Lycopene, and α- and β-Carotene Simultaneously Determined in Plasma by Isocratic Liquid Chromatography

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Retinol, α-tocopherol, lycopene, and α- and β-carotene can be simultaneously determined in human plasma by reversed-phase liquid chromatography. Plasma—0.5 mL plus added internal standard, retinyl acetate—is deproteinized with 0.5 mL of ethanol, then extracted with 1.0 mL of petroleum ether. The organic layer is removed and evaporated, the residue is redissolved in 0.25 mL of ethanol, and 8-μL samples are injected into a 60 × 4.6 mm column of Hypersil ODS 3-μm particles at 35 °C. An isocratic methanol mobile phase, flow rate 0.9 mL/min, is used for the 9-min run. Retinol and retinyl acetate are monitored at 305 nm, the tocopherols at 292 nm, and the carotenoids at 460 nm. Between-run CVs were 3.1, 6.9, 6.1, and 6.5% for retinol, α-tocopherol, lycopene, and β-carotene, respectively. Small sample requirement, simplicity of extraction, short run time, and good reproducibility make this procedure ideal for clinical or research use.

Additional Keyphrases: *vitamin A · vitamin E · nutrition effect of various anticoagulants*

Clinical interest in evaluation of vitamin A and vitamin E nutriture has increased in recent years, mainly owing to the possible roles of retinol (vitamin A), β-carotene, and α-tocopherol (vitamin E) in decreasing the risk of cancer (*1*). These vitamins are also important in premature infants and patients who are receiving long-term total parenteral nutrition. Thus, a rapid, sensitive, relatively simple, and specific method for determination of these vitamins in plasma is desirable. Conventional colorimetric or fluorimetric methods for determining retinol, carotene, or α-tocopherol (*2–3*) are either nonspecific, time consuming, or insensitive.

Many of these problems are eliminated when these micronutrients are determined by "high-performance" liquid chromatography (LC). Several LC procedures have already been described for determination of retinol and α-tocopherol, either separately (*4–6*) or simultaneously (*7–10*). Additional procedures can separate lycopene, α-carotene, and β-carotene from other carotenoids (*11–14*). Nevertheless, an assay that could resolve and quantify all of these compounds simultaneously would be highly desirable from the standpoint of clinical assessment and studies of their metabolism.

Procedures recently described for determination of retinol, α-tocopherol, β-carotene, α-carotene, and lycopene in a single run (*15, 16*) involve either a multiple-solvent gradient system or complex solvent mixtures and 15 to 30 min of analysis times. Here we describe an isocratic LC procedure in which only methanol is used in the mobile phase. With it, retinol, α-tocopherol, lycopene, α-carotene, and β-carotene extracted from 0.5 mL of plasma can be resolved and estimated in a single 9-min run.

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Materials and Methods

Apparatus. The chromatographic instrumentation was a Model 1090 LC System (Hewlett-Packard, Avondale, PA)[1] with a variable-wavelength diode-array detector, attached to the Hewlett-Packard 3388 integration system and controlled by a HP85B computer. For all analyses we used a 60 × 4.6 mm column of 3-μm Hypersil ODS (Hewlett-Packard), operated at 35 °C. The mobile phase was methanol, the flow rate 0.9 mL/min.

Reagents. Retinol, retinyl acetate, α-tocopherol, lycopene, α-carotene, and β-carotene were all from Sigma Chemical Co., St. Louis, MO. All solvents were "HPLC" grade.

We dissolved the standards individually in chloroform. We then further diluted them in ethanol to obtain approximate final concentrations of 1 mg/L for retinol and the carotenoids, 40 mg/L for α-tocopherol. The actual concentrations of the standards were determined from the absorbances and the absorptivities (in $L \cdot mol^{-1} \cdot cm^{-1}$): 1780 at 325 nm for retinol, 75.8 at 292 nm for α-tocopherol, 3450 at 472 nm for lycopene, 2800 at 444 nm for α-carotene, and 2396 at 465 nm for β-carotene.

Procedures. All procedures were carried out in a laboratory equipped with yellow lights that did not include the ultraviolet end of the spectrum. This minimized light-induced degradation of the vitamins. We added 0.5 mL of ethanol that contained 0.6 μg of retinyl acetate per milliliter to 0.5 mL of plasma in a 10 × 75 mm glass tube, vortex-mixed the mixture for 1.0 min, added 1.0 mL of petroleum ether (bp 36.6–56.6 °C), and vortex-mixed again for 1 min. After centrifuging (600 × *g*, 5 min) we transferred the supernate to a separate tube with a Pasteur pipet and evaporated it to dryness in a 60 °C water bath, under a stream of nitrogen. We then redissolved the extract in 0.25 mL of ethanol,[2] vortex-mixed for 2.0 min, then filtered the mixture through a Millipore filter (no. SFH004NS; Millipore Corp., Bedford, MA) into injection vials. These were placed in the autosampler and 8 μL was injected into the chromatograph, which then was eluted as described above.

We programmed the diode array detector as follows: 0 to 1.6 min at 305 ± 40 nm, to determine retinol and retinol acetate; from 1.6 min to 2.5 min at 290 ± 10 nm, to determine α- and γ-tocopherol; and from 2.5 to 9.0 min at 460 ± 25 nm, to determine the carotenoids. The reference wavelength was 575 nm throughout the run. The baseline was automatically adjusted to zero with each wavelength change. Four minutes after injection, the attenuation was increased fourfold.

[1] Mention of a trademark or proprietary product does not constitute a guarantee or warranty by the U.S. Dept. of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

[2] The samples were redissolved in ethanol, because the fat-soluble vitamins and carotenoids are much more soluble in ethanol than in (more polar) methanol.

Peak heights were compared with those for known standards, run with retinyl acetate as the internal standard. The concentrations of the analytes were determined by the peak-height ratio method (*10*). Samples for assessment of analytical recovery were run by adding known standards, dissolved in 0.5 mL of ethanol, to 0.5 mL of plasma before extraction with petroleum ether, as follows: retinol, 0.14 to 0.68 μg; α-tocopherol, 0.985 to 4.925 μg; lycopene, 0.14 to 0.25 μg; and β-carotene, 0.14 to 0.25 μg.

Effect of anticoagulants. A 30-mL specimen of blood was collected from each of six different individuals into a plastic syringe, for use in testing the effects of different anticoagulants on results for these fat-soluble vitamins. Each sample was divided into five aliquots, which were handled as follows. One was allowed to clot and the serum obtained. The others were added to Vacutainer Tubes containing heparin, citrate, EDTA, and oxalate, respectively, and plasma was collected for analysis.

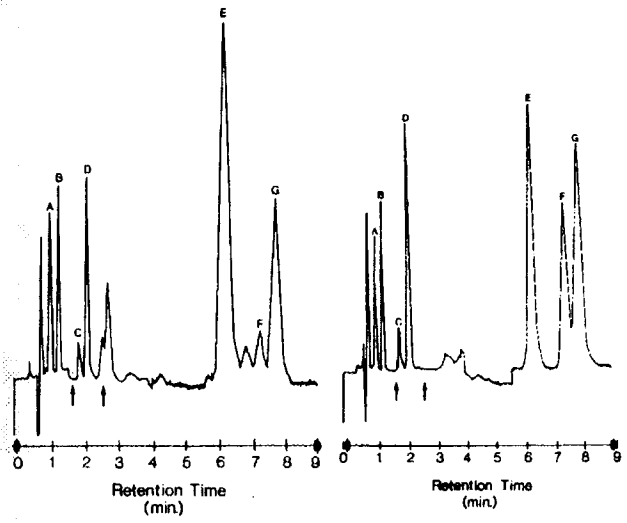

Fig. 1. Chromatograms of fat-soluble vitamins extracted from 0.5 mL of (*A, left*) plasma and (*B, right*) of standards (see text)

Peaks are: *A*, retinol; *B*, retinyl acetate; *C*, γ-tocopherol; *D*, α-tocopherol; *E*, lycopene; *F*, α-carotene; and *G*, β-carotene. Peaks *A* and *B* were measured at 305 nm, peaks *C* and *D* at 290 nm, and peaks *E* through *G* at 460 nm. The *arrows* indicate the wavelength changes. Response set at 0.012 *A* full-scale for peaks *A* through *D*, and 0.003 *A* for peaks *E* through *G*

Results and Discussion

Figure 1*A* depicts a typical chromatogram of fat-soluble vitamins from plasma. It compares well with a chromatogram of pure standards (Figure 1*B*). Comparisons of retention times and spectra of fractions with those of pure standards established the peak identities. According to a recent report (*14*), the two unidentified peaks after α-tocopherol may be precryptoxanthin and cryptoxanthin.

Analytical recoveries of different concentrations of standards added to five different plasma samples before extraction averaged 99.1 (SD 1.38), 101.7 (8.9), 96.5 (6.6), and 98.8 (1.9) percent for retinol, α-tocopherol, lycopene, and β-carotene, respectively. The corresponding correlation coefficients for expected vs found were respectively 0.986, 0.995, 0.983, and 0.998 over these two- to fivefold concentration ranges. The detection limits were 0.1, 0.3, and 0.02 mg/L for retinol, α-tocopherol, and the carotenoids, respectively.

Table 1 summarizes within-run and day-to-day CVs. We have successfully used smaller volumes of serum (down to 200 μL) with proportional reductions in the other reagents without affecting recovery, precision, or final result.

Effects of different anticoagulants are summarized in Table 2. Values obtained with heparin present in the sample were not significantly ($p > 0.05$) different from values for serum. Citrate significantly depressed the values for apparent retinol and α-tocopherol in plasma; oxalate significantly depressed only the α-tocopherol concentrations. EDTA and oxalate produced insignificantly ($p > 0.05$) lower values for retinol and α-carotene. Insignificantly lower values for α-tocopherol were also seen when EDTA was used as anticoagulant. None of the anticoagulants affected values obtained for either lycopene or β-carotene. These results are consistent with those found by McClean et al. (*18*), who reported lower results for retinol when either EDTA or oxalate was used as anticoagulant. Nierenberg (*19*) noted that under the condition of his assay EDTA, oxalate, or citrate adversely affected values for β-carotene, and he suggested that these compounds may catalyze reactions resulting in the isomerization or oxidation of retinol and β-carotene. Our results indicate that the various anticoagulants affect α-tocopherol in a similar manner. Therefore, we recommend either serum or heparinized plasma in this assay.

As recommended by others (*19, 20*), we tested the useful-

Table 1. Within-Run and Day-to-Day Variation[a]

	Retinol	α-Tocopherol	Lycopene	β-Carotene
Day 1				
Mean[b]	0.485 (0.016)	7.3 (0.12)	0.418 (0.017)	0.109 (0.003)
CV, %	3.3	1.6	4.1	2.75
Day 2				
Mean	0.502 (0.03)	7.6 (0.16)	0.432 (0.01)	0.112 (0.006)
CV, %	6.0	2.1	2.3	5.4
Day 3				
Mean	0.466 (0.01)	6.9 (0.15)	0.408 (0.01)	0.100 (0.005)
CV, %	2.1	2.2	2.5	5.0
Day 4				
Mean	0.485 (0.03)	8.3 (0.17)	0.476 (0.02)	0.120 (0.007)
CV, %	6.3	2.0	4.2	5.8
Day 5				
Mean	0.503 (0.03)	7.8 (0.4)	0.446 (0.03)	0.112 (0.005)
CV, %	6.0	5.1	6.7	4.5
Overall (day-to-day)				
Mean	0.488 (0.015)	7.58 (0.53)	0.436 (0.027)	0.111 (0.007)
CV, %	3.1	6.9	6.1	6.5

[a] Variability of a single plasma pool sample. Four replicates, determined on each of five separate days. α-Carotene was not detected in this sample.
[b] Mean (and SD), in mg/L.

Table 2. Effect of Anticoagulants on Values Observed for Fat-Soluble Vitamins[a]

	Retinol	α-Tocopherol	Lycopene	α-Carotene[b]	β-Carotene
	Mean concn, mg/L (and SD)				
Serum	0.51 (0.14)	9.10 (1.18)	0.49 (0.12)	0.14 (0.06)	0.16 (0.05)
Heparin	0.51 (0.17)	8.70 (1.67)	0.45 (0.11)	0.14 (0.13)	0.14 (0.05)
Citrate	0.40 (0.08)[c]	7.06 (0.70)[d]	0.43 (0.08)	0.11 (0.003)	0.15 (0.04)
EDTA	0.45 (0.14)	8.90 (1.18)	0.47 (0.14)	0.09 (0.09)	0.16 (0.06)
Oxalate	0.43 (0.12)	7.40 (0.86)[e]	0.45 (0.12)	0.08 (0.06)	0.14 (0.04)

[a] Blood from six individuals; each sample was divided into five aliquots, which were treated with the anticoagulants indicated.
[b] α-Carotene was not detectable on 3/6 samples of serum, 4/6 samples with heparin, 3/6 samples with citrate, 1/6 samples with EDTA, and 4/6 samples with oxalate.
[c] Significantly lower than serum or heparin ($p < 0.04$) by Scheffé contrasts (*21*).
[d] Significantly lower than serum, heparin, or EDTA ($p < 0.001$).
[e] Significantly lower than serum, heparin, or EDTA ($p < 0.01$).

Table 3. Fat-Soluble Vitamins and Carotenes in Plasma from 12 Healthy Persons

	Retinol	α-Tocopherol	Lycopene	α-Carotene	β-Carotene
	Concn, mg/L				
	0.620	9.6	0.655	0.107	0.230
	0.611	12.0	0.370	0.082	0.177
	0.531	10.7	0.197	0.037	0.077
	0.445	8.2	0.510	0.108	0.196
	0.591	11.5	0.489	0.144	0.337
	0.637	13.5	0.843	0.113	0.296
	0.393	10.5	0.674	0.029	0.192
	0.450	11.3	0.155	nd[a]	0.215
	0.532	11.5	0.276	0.041	0.102
	0.850	10.5	0.356	nd	0.094
	0.679	7.3	0.241	nd	0.189
	0.696	8.8	0.344	nd	0.076
Mean	0.586	9.6	0.426	0.055	0.182
SD	0.127	3.3	0.212	0.053	0.084

[a] nd, not detectable, <20 μg/L.

ness of BHT (butylated hydroxytoluene) for stabilizing the fat-soluble vitamins and carotenoids during the extraction and drying steps. When 38 mg of BHT was added to the ethanol per liter before the deproteinization and extraction steps, we found no differences in the values for α-tocopherol or any of the carotenoids as compared with aliquots of the same samples extracted without added BHT. Under the conditions of our assay, BHT is eluted near the retinol peak and thus interferes with its determination. Nevertheless, addition of an antioxidant such as BHT may be necessary in systems that contain free-radical-producing or oxidizing agents such as tetrahydrofuran or $HClO_4$ (*19*).

Values for plasma from 12 apparently healthy adults (Table 3) are similar to those reported by others for retinol (*15, 17*), α-tocopherol (*8, 15*), lycopene (*14, 15*), α-carotene (*14*), and β-carotene (*14, 15, 19*).

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