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Short Communication

Rapid HPLC simultaneous determination of fat-soluble vitamins, including carotenoids, in human serum*

L. YAKUSHINA† and A. TARANOVA

Institute of Nutrition of Russian Academy of Medical Sciences, 109240 Moscow, Ustinsky pr., 2/14, Russia

Keywords: *Fat-soluble vitamins; β-carotene; carotenoids; biological fluids; non-aqueous reversed-phase LC; simultaneous determination.*

Introduction

Reliable, sensitive and rapid methods of determination of vitamins in biological fluids are essential for the evaluation of human vitamin status as well as of vitamin bioavailability. Consequently, it is very important to be able to determine simultaneously vitamins such as retinol, tocopherols and their ethers, β-carotene and carotenoids in a single run. The complexity of the simultaneous determination of fat-soluble vitamins is caused by differences in their spectral characteristics (absorption maxima vary in the range of 292–450 nm); for the simultaneous determination of these vitamins selective chromatographic media and conditions are needed. For example, retinyl palmitate was eluted simultaneously with β-carotene while some carotenoids were eluted with retinol, tocopherols and their ethers. To solve this problem the use of several columns connected in parallel under the optimal conditions for each vitamin and with several detectors was suggested [1–3]. Another model reported earlier by workers in this laboratory [4, 5] suggested the use of one detector with programmed wavelength change and a single column. However, it was necessary to analyze the same sample twice: first to determine β-carotene and carotenoids at 450 nm; and second to determine the remainder of the fat-soluble vitamins with the use of a variable-wavelength detector. The aim of the present work was to determine retinol, tocopherols and their ethers as well as β-carotene and the main carotenoids in a serum sample in a single run and to increase the productivity of the analyses.

Experimental

Sample preparation

200 μl of serum was transferred by pipette into a 4-ml centrifuge tube and 200 μl of a methanolic solution of tocopheryl acetate as the internal standard (65 μg dl^{-1}) was added; the mixture was shaken in a vortex mixer for 30 s and 200 μl of *n*-hexane was added. The tube was sealed with a polyethylene stopper, shaken for 15 min and centrifuged at 3000 rpm for 10 min. 120 μl of the hexane extract was transferred to a 1.5-ml microcentrifuge tube and evaporated under nitrogen to remove the solvent. The residue was redissolved in 20 μl of dichloromethane and 100 μl of acetonitrile–methanol (1:1, v/v) was added. Of this sample solution, 50 μl was injected on to the column.

Equipment and operating conditions

The chromatographic system (Jasco, Japan) included an 880-PU pump; two sequentially

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†Author to whom correspondence should be addressed.

connected detectors were used: an 870-UV spectrophotometer at 450 nm for determination of the carotenoids and an 821-FP fluorescence spectrophotometer with a programmable variable wavelength for the assay of retinol, tocopherols and their ethers. Detection: excitation wavelength 325 nm, emission 480 nm (for retinol); after 5 min of the run the excitation wavelength was changed to 295 nm and the emission wavelength was changed to 330 nm (for γ- and α-tocopherols and tocopheryl acetate); after 15 min the exication wavelength was changed to 325 nm, and emission wavelength was 480 nm (for retinyl palmitate). The column (150 × 4 mm i.d.) was packed with Nucleosil C18, 5 μm (Elsico, Russia) and the mobile phase was acetonitrile–methanol–dichloromethane (50:45:5, v/v/v), at a flow-rate of 0.7 ml min^{-1}. The volume of the injection loop was 50 μl.

Results and Discussion

Several HPLC techniques for simultaneous determination of fat-soluble vitamins which involve normal (NP)- or reversed-phase (RP) systems have been described previously. The use of NP conditions may give rise to overestimation of the β-carotene concentration; under these conditions β-carotene was eluted very close to the dead volume of the column. However, the procedure of sample preparation was very simple under these conditions and comprised deproteination with methanol, subsequent extraction of fat-soluble vitamins with *n*-hexane and direct injection of the hexane extract on to the column. Several researchers have developed RP HPLC methods with a variety of organic solvents and various HPLC columns [6–9].

Non-aqueous RP conditions for the quantitative determination of β-carotene and other fat-soluble vitamins were chosen for the present work. In a procedure of this type the solvent used for extraction of the serum had to be removed and the extract transferred to a solvent compatible with the stationary phase of the column. To achieve these conditions, the residue after evaporation under nitrogen was redissolved in 20 μl of dichloromethane (to dissolve β-carotene and carotenoids) and then acetonitrile–methanol (1:1, v/v) was added). In this case an internal standard, tocopheryl acetate had to be used to obtain quantitative results.

Typical chromatograms of human serum vitamins are shown in Figs 1 and 2. Perfect separation of the main carotenoids as well as retinol, γ- and α-tocopherols, tocopheryl acetate (internal standard) and retinyl palmitate was obtained. The principal carotenoids in the chromatogram shown in Fig. 2 have been

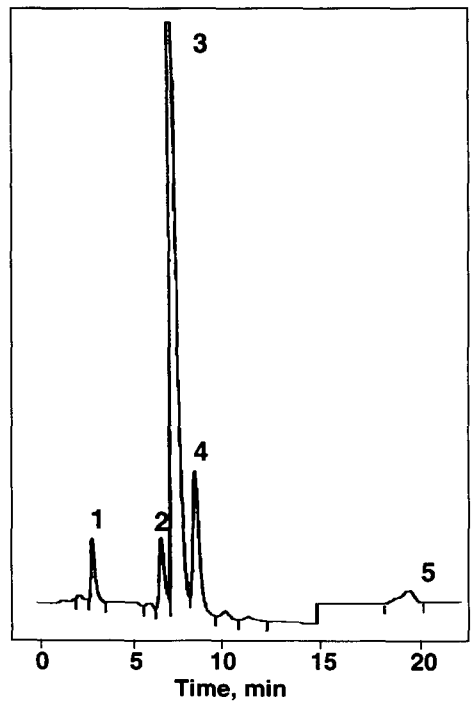

Figure 1
Fat-soluble vitamins of human serum. Chromatographic conditions: column (150 × 4 mm, i.d.) with Nucleosil C_{18}, 5 μm (Elsico, Russia); eluent: acetonitrile–methaol–dichloromethane (50:45:5, v/v/v), 0.7 ml min^{-1}; fluorescence detector 821-FP (Jasco, Japan); initial: λ_{ex} 325 min, λ_{em} 480 nm; after 5 min: λ_{ex} 295 nm, λ_{em} 330 nm; after 15 min; λ_{ex} 325 nm, λ_{em} 480 nm. Key: (1) retinol; (2) γ-tocopherol; (3) α-tocopherol; (4) tocopheryl acetate; (5) retinyl palmitate.

Table 1
The retention time of fat-soluble vitamins and carotenoids*

Vitamins and carotenoids	Retention time (min)
Retinol	2.9
γ-Tocopherol	6.5
α-Tocopherol	7.3
Tocopheryl acetate	9.3
Retinyl palmitate	19.3
Lutein/zeaxanthin	3.5
Cryptoxanthin	7.7
Lycopene	11.5
α-Carotene	17.1
β-Cartene (all trans-)	18.4
β-Cartene (15,15′-*cis*-)	19.5

*For chromatographic conditions, see text.

Table 2
Within-run and between-run precision of the method

Vitamins and carotenoids	Serum	Within-run *n*	Within-run Mean ± SD (μg dl^{-1})	Within-run RSD	Between-run *n*	Between-run Mean ± SD (μg dl^{-1})	Between-run RSD
Retinol	A	20	53.8 ± 2.0	3.7	26	53.1 ± 1.9	3.5
	B	16	49.4 ± 1.3	2.7	12	47.2 ± 1.9	4.1
γ-Tocopherol*	A		— —	—	—	— —	
	B	16	0.11 ± 0.01	4.72	12	0.11 ± 0.01	4.88
α-Tocopherol*	A	22	0.82 ± 0.04	4.22	21	0.83 ± 0.06	6.99
	B	16	1.16 ± 0.04	3.24	12	1.16 ± 0.04	3.43
Retinyl palmitate	A	22	17.7 ± 1.8	10.2	15	21.7 ± 2.4	11.2
	B	15	200.8 ± 8.5	4.2	12	203.3 ± 12.1	6.0
Lutein/zeaxanthin	A	22	10.6 ± 0.6	4.4	22	10.0 ± 1.1	11.0
	B	11	14.8 ± 0.3	2.1	12	14.2 ± 0.9	6.3
Cryptoxanthin	A	20	1.0 ± 0.04	4.43	21	1.2 ± 0.1	10.0
	B	10	6.1 ± 0.4	7.0	10	6.5 ± 0.4	6.4
Lycopene	A	21	4.8 ± 0.4	8.1	26	4.5 ± 0.4	8.9
	B	14	23.2 ± 1.3	5.6	12	21.6 ± 1.3	6.2
α-Carotene	A	23	2.4 ± 0.1	5.6	26	2.0 ± 0.3	12.4
	B	12	5.2 ± 0.3	5.6	12	5.0 ± 0.2	3.3
β-Carotene	A	22	6.2 ± 0.4	6.3	23	6.7 ± 0.5	6.9
	B	10	16.1 ± 0.5	3.2	12	16.4 ± 0.7	4.1
Unidentified carotenoids	A	24	2.2 ± 0.1	6.4	25	2.2 ± 0.2	9.5
	B	8	10.5 ± 1.1	10.2	10	11.3 ± 0.7	6.3

*Concentrations of tocopherols expressed as mg dl^{-1}.

Table 3
Recovery of vitamins and carotenoids from serum

Vitamins and carotenoids	Recovery, % (mean ± SD)
Retinol	92 ± 2.1
α-Tocopherol	97 ± 3.7
Tocopheryl acetate	99 ± 2.5
Retinyl palmitate	95 ± 4.8
Lutein	93 ± 3.6
Lycopene	105 ± 3.2
α-Carotene	98 ± 4.5
β-Carotene	102 ± 3.3

identified as 1 — lutein/zeaxanthin (these peaks coincide), 3 — cryptoxanthin, 4 — lycopene, 6 — α-carotene, 7 — all-trans-β-carotene and 8 — 15,15′-*cis*-β-carotene. Identification of the peaks was performed by comparing their retention times and spectral ratios with those of the corresponding vitamin standard solutions. Retention times of fat-soluble vitamins and carotenoids are presented in the Table 1. Two serum samples (A and B) with different concentrations of vitamins were analysed 10–20 times on the same day or 10–25 times on the consecutive days, in order to calculate the within-run and the between-run precision of the method and the results are

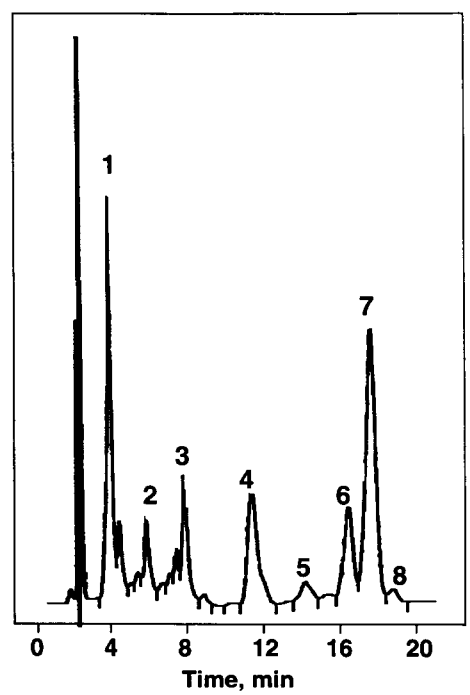

Figure 2
Principal carotenoids in human serum. For chromatographic conditions, see Fig. 1. Detector: UV-spectrophotometer 870-UV (Jasco, Japan); λ 450 nm. Key: (1) lutein/zeaxanthin; (2) and (5), unidentified; (3) cryptoxanthin; (4) lycopene; (6) α-carotene; (7) all trans-β-carotene; (8) 15,15′-*cis*-β-carotene.

shown in Table 2. The recoveries of the vitamins and carotenoids were determined by adding various amounts of standards of retinol, α-tocopherol, retinyl palmitate, lutein, lycopene, α- and β-carotenes to pooled serum. Recoveries were calculated by comparing the peak areas of spiked samples to those of the standards (Table 3). All experiments were carried out in triplicate on different days.

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