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HPLC method for the simultaneous determination of beta-carotene, retinol and alpha-tocopherol in serum*

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Abstract: A method is described for the simultaneous analysis of α-tocopherol, β-carotene, and retinol in *n*-hexane extracts of serum. The technique uses normal-phase HPLC in a silica gel column with *n*-hexane-2-propanol (97:3, v/v) as the mobile phase. The compounds are eluted from the column in the order α-tocopherol, β-carotene, tocol (internal standard) and retinol. The compounds are quantified by use of the following detectors connected in series: retinol and tocol by a spectrophotometer, β-carotene by a filter photometer, and α-tocopherol and tocol by a spectrophotofluorimeter. The time taken for separation is less than 6 min but a further 14 min is allowed for elution of the more polar compounds before the next run. With an automatic injector, the method is suitable for the analysis of a large number of samples because of the relatively short time for analysis and the small volume (0.2 ml) of serum required. The method has been used in a study of the relationships between serum levels of the fat-soluble vitamins and subsequent cancer in 2332 subjects.

Keywords: *β-Carotene; retinol; α-tocopherol; normal-phase HPLC; serum.*

Introduction

In recent years HPLC methods have been published [1–3] for the simultaneous determination of vitamins A and E in serum or plasma using reversed-phase columns and a single UV-detector. Only one paper [3] describes the simultaneous analysis of β-carotene. A method was required to enable these three compounds to be determined in more than two thousand samples. For these purposes, an adsorption column proved to be superior to a reversed-phase column. To increase sensitivity and specificity, a separate detection device was used for each of the three compounds.

Experimental

Reagents and chemicals

n-Hexane (chromatography grade) was purchased from Merck (Darmstadt, F.R.G.);

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2-propanol (HPLC grade) from Rathburn Chemicals (Walerburn, Great Britain); ethanol (spectrophotometric grade) from ALKO (Rajamäki, Finland); dl-tocol from Hoffman-La Roche (Basle, Switzerland); β-carotene (from carrot, crystalline, type IV) and retinol (crystalline, synthetic type X) from Sigma (St. Louis, MO, U.S.A.); and d-α-tocopherol (analytical grade) from Eastman Kodak (Rochester, NY, U.S.A.).

Instrumentation and the chromatographic analysis

Normal-phase HPLC was performed using a Waters 6000A solvent delivery system (Waters Assoc., Milford, MA, U.S.A.) with a Rheodyne loop injector, or with a Waters WISP 710 B, an automatic injector and a 10-μm silica gel μPorasil column No. 27477 (Waters), preceded by a guard column packed with Corasil II (Waters). The sample or standard dissolved in 35 μl of the eluent was injected onto the column. The column was eluted with *n*-hexane-2-propanol (97:3, v/v) at a flow rate of 1.0 ml/min (pressure 600 psi). The absorbances of retinol and the internal standard, dl-tocol, were monitored at 305 nm with a Waters Lambda Max Model 480 LC spectrophotometer (detector sensitivity, 0.02) and that of β-carotene at 436 nm with a Waters Model 440 absorbance detector (detector sensitivities, 0.001, 0.02 and 0.05). Absorbances were recorded by a double-pen recorder, Waters Data Module. The fluorescence intensities of α-tocopherol and the internal standard, dl-tocol, were monitored using a Perkin–Elmer Model LS-4 fluorescence spectrophotometer (Norwalk, CT, U.S.A.) (detector set at 0.12) with excitation at 292 nm and emission at 324 nm, and were recorded by a Servocor 120 recorder (BBC Goerz Metrawatt).

Preparation of standard solutions

Stock solutions had the following concentrations: β-carotene 5.60 mg/100 ml of *n*-hexane; retinol 15.54 mg/100 ml of ethanol; dl-tocol 25.35 mg/100 ml of ethanol; and α-tocopherol 68.95 mg/100 ml of *n*-hexane. The concentrations of the working standard solutions were checked using the following values for $A^{1\%}_{1cm}$: 1835 for β-carotene at 325 nm, and 71 for α-tocopherol at 292 nm.

All vitamin standards were protected from light and stored in nitrogen at −20°C, except α-tocopherol which was stored at +4°C. Working standards were prepared and concentrations were confirmed monthly from stock standards. The standards were analysed weekly by HPLC except that for α-tocopherol, which was analysed twice a week.

Standardization

Three working standards were prepared with increasing concentrations of β-carotene, retinol and α-tocopherol and a fixed concentration of the internal standard, dl-tocol, and were analysed by HPLC. Peak heights were measured. The ratio of the height of the retinol peak to that of the internal standard, tocol, at 305 nm was calculated and so were the ratio of the α-carotene peak at 436 nm to the peak of tocol at 305 nm, and the ratio of the fluorescence peaks of α-tocopherol and tocol. The ratios were plotted against the corresponding absolute concentrations of the standards. The graphs showed good linearity in the following concentration ranges: β-carotene, 120–240 μg l^{-1} (detector sensitivity, 0.010), 240–480 μg l^{-1} (0.020) and 300–1200 μg l^{-1} (0.050); retinol, 450–1130 μg l^{-1}; α-tocopherol, 400–1800 μg/100 ml. The concentrations found in serum fitted in these ranges. In typical results from standardizations done every week, the coefficients m and c in the equation $y = mx + c$ were: β-carotene 132.78 and

1.56; retinol 132.78 and 23.26; and α-tocopherol 651.55 and 64.67, respectively. All plots had correlation coefficients of 0.999–1.000.

Preparation of samples

Blood samples were obtained by venepuncture. The serum was removed, frozen and stored at −20°C until removed for analysis. The samples were thawed in a refrigerator and mixed for 15 min with a Heidolph Reax mixer. Aliquots of 200 μl of serum were mixed with 200 μl of ethanol (99.5%) containing 25 μl of dl-tocol by means of a vortex mixer for a few seconds. After the addition of 500 μl of *n*-hexane and mixing in a vortex mixer for 1 min, the samples were centrifuged for 10 min at 1500 **g**. The organic layer was removed. The residue was washed once with 500 μl of *n*-hexane and centrifuged. The combined supernatants were evaporated to dryness in a stream of nitrogen. The residue was dissolved in 200 μl of the HPLC eluent, *n*-hexane-2-propanol (97:3, v/v). A 35-μl volume of the extract was injected onto the μPorasil column. To ensure that all the more polar compounds had been eluted from the column, the whole run time was 20 min although the actual separation was effected in about 5 min.

Results and Discussion

Typical chromatograms obtained from a single run of an extract from human serum are shown in Fig. 1. In Fig. 1A are shown elution profiles of β-carotene (right) and

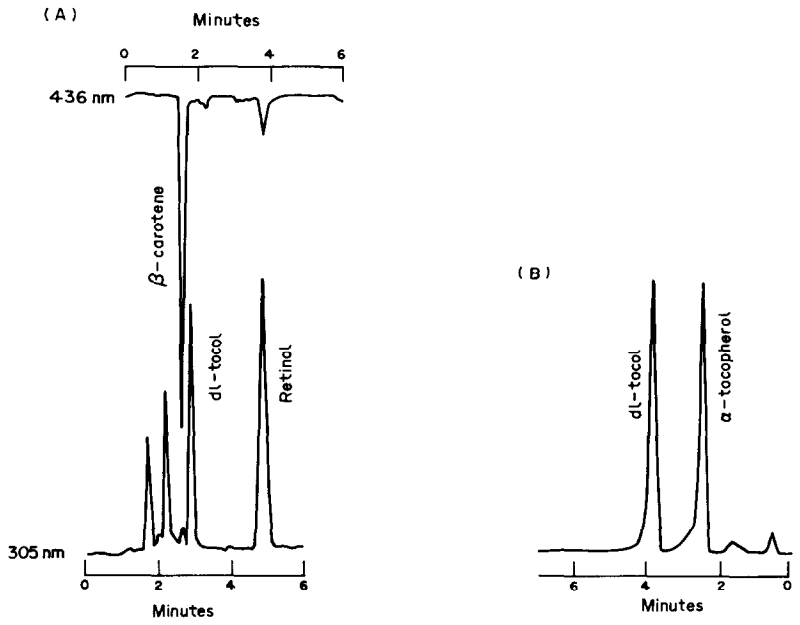

Figure 1
Typical HPLC patterns of an *n*-hexane extract of a freshly obtained normal serum sample. Column: 200 × 3.9 mm, i.d. packed with 10-μm μPorasil, preceded by a guard column of Corasil II. Eluent: *n*-hexane-2-propanol (97:3, v/v). Flow rate: 1.0 ml min^{-1}. Detection: A. UV-detector at 305 nm for dl-tocol and retinol (left) and at 436 nm for β-carotene. B. Fluorimetric detector for dl-tocol and α-tocopherol (excitation at 292 nm and emission at 324 nm). The tubing between the UV-spectrophotometer and spectrophotofluorimeter causes a delay in retention time of tocol between A and B.

Table 1
Reproducibility of the results of serum vitamin determinations*

Sex	Compound	No. of days	Mean of morning results	Difference between morning and evening results	*P* value for the difference	Relative standard deviation (%) within day	Relative standard deviation (%) day-to-day
Men	α-tocopherol	82	580	+20	<0.001	3.2	6.0
	β-carotene	82	201	0	0.612	2.3	8.6
	retinol	82	747	−4	0.435	5.0	5.7
Women	α-tocopherol	98	611	+11	<0.001	2.1	5.3
	β-carotene	98	220	−3	0.072	2.7	10.9
	retinol	98	735	−13	<0.001	2.6	4.9

* Results in μg/100 ml for α-tocopherol and μg l^{-1} for β-carotene and retinol.

retinol (left) with dl-tocol as the internal standard. Figure 1B demonstrates the detection of α-tocopherol using the same internal standard. Each peak was well separated from the others. Negative peaks were not observed. Some samples stored at −20°C showed an elevation of the baseline at 305 nm, which interfered with the measurement of α-tocopherol. Because of this problem, α-tocopherol was determined fluorimetrically.

The same control serum pool was analysed twice a day, once in the morning and once in the evening. The within-day and day-to-day variations were determined from these data (Table 1). Relative standard deviations of the results ranged from 2.1 to 10.9%. The within-day variation was smaller than the day-to-day variation. The highest day-to-day variation was for β-carotene. The level of α-tocopherol was statistically significantly higher in the morning than in the evening. This was apparently because all samples to be analysed during the day were thawed at the same time in the morning. The standards were handled in the same way. β-Carotene showed no systematic within-day changes.

The method has been successfully used in connection with a study on the association between the serum levels of these vitamins and subsequent cancer in a total of 2332 subjects [4, 5].

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