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Short Communication

Simple determination of retinol, α-tocopherol and carotenoids (lutein, all-*trans*-lycopene, α- and β-carotenes) in human plasma by isocratic liquid chromatography

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Abstract

Retinol, α-tocopherol, lutein, all-*trans*-lycopene, and α- and β-carotenes can be simultaneously determined in human plasma by reversed-phase liquid chromatography with a programmable and variable UV wavelength detector. Plasma (200 μl) is deproteinized with 200 μl of ethanol containing retinyl acetate, as internal standard, then extracted with 1.0 ml of *n*-hexane–2,6-di-*tert*.-butyl-*p*-cresol (BHT). The organic layer is removed and evaporated and the residue is dissolved in 100 μl of a mixture of acetonitrile–tetrahydrofuran–methanol (68:22:7, v/v/v). Aliquots (15 μl) are injected onto a 250 × 4.6 mm I.D. column of Nucleosil 100-5 C_{18} with a pre-column of Lichrosorb RP-18, 7 μm, 15 × 3.2 mm I.D. A mobile phase of acetonitrile–tetrahydrofuran–methanol (68:22:7, v/v/v), adjusted to 100 (v/v) with 1% ammonium acetate, at a flow-rate of 1.5 ml/min is used. Usual run time is 15 min. Retinol and retinyl acetate are monitored at 325 nm, tocopherol at 290 nm, lycopene at 470 nm, lutein and α- and β-carotenes at 450 nm. The intra-batch coefficients of variation (C.V.) were 2.5, 2.2, 2.4, 5.8, 5.1 and 4.7% for retinol, α-tocopherol, lutein, *trans*-lycopene, α- and β-carotenes, respectively. The inter-batch C.V.s of experiments performed on 30 different days over 12 weeks were 5.7, 3.9, 4.5, 10.9, 11.3 and 10.5%, respectively.

1. Introduction

Antioxidant vitamins, *i.e.* vitamins A, C, E and carotenoids, have been suggested to play an important role in cardiovascular disease and cancer [1–6]. It is, therefore, not surprising that antioxidant vitamins, especially fat-soluble vitamins, are considered as useful clinical parameters related to myocardial infarction and cancer incidence.

Increased interest in these vitamins has resulted in the introduction of several methods for their analysis. Liquid chromatography (LC) is mostly used for determining these micronutrients. LC procedures have already been described for determination of retinol and α-tocopherol simultaneously in biological fluids [7–9]. Additional procedures can separate carotenoids [10–14]. Procedures recently described for determination of the combined analysis of vitamins A, E and carotenoids in plasma generally employed two detectors, a fluorometer and a spectrophotometer or more than one HPLC lines [15–20].

In this paper, we describe a relatively quick, highly sensitive method for the simultaneous quantitation of retinol, α-tocopherol, lutein, lycopene, and α- and β-carotene in human

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plasma using isocratic reversed-phase liquid chromatography. A UV-Vis programmable wavelength spectrophotometer has been used as detector. Several thousand plasma samples have been analyzed using this method.

2. Experimental

Low-actinic glassware was used for all preparations.

2.1. Materials

Retinol, retinyl acetate and α-tocopherol were obtained from Fluka (Buchs, Switzerland). α-, δ- and γ-Tocopherols were purchased from Eastman Kodak (Rochester, NY, USA). Lutein was obtained from Sigma Chemical (Buchs, Switzerland). Lycopene, β-cryptoxanthin, α- and β-carotenes were gifts from Hoffmann-La Roche (Basel, Switzerland). HPLC-grade acetonitrile, methanol, and *n*-hexane were obtained from Riedel–de Haën (Seelze, Germany). Absolute ethanol, ammonium acetate, 2,6-di-*tert.*-butyl-*p*-cresol (BHT) were obtained from Merck (Darmstadt, Germany). Tetrahydrofuran, analytical grade, was purchased from SDS (Peypin, France) and it was free from peroxide (tested by Jorissen reagent) and was used before storage deadline.

2.2. Solutions

n-Hexane–BHT solution (0.4 g BHT in 100 ml *n*-hexane). Acetonitrile–tetrahydrofuran–methanol composition was 68:22:7 (v/v/v).

2.3. Calibration solutions

Individual stock solutions were prepared using pure standard retinol, retinyl acetate, α-tocopherol, lutein, all-*trans*-lycopene, and α- or β-carotenes and stored at −20°C. Retinol, retinyl acetate and α-tocopherol are soluble in hexane. Lycopene and the two carotenes were dissolved in small quantities of dichloromethane, and the solutions were then diluted with *n*-hexane–BHT. The concentrations of these standard dilutions were determined from published absorption coefficients $E^{1\%}$, cm^{-1} using either ethanol or hexane as solvent: retinol, 1835 at 325 nm (ethanol); retinyl acetate, 1550 at 326 nm (ethanol); tocopherol, 71 at 292 nm (ethanol); lutein, 2550 at 445 nm (ethanol); lycopene, 3450 at 472 nm (hexane); α-carotene, 2725 at 446 nm (hexane); and β-carotene, 2592 at 450 nm (hexane). The stock solutions were stable for approximately two months, except for the lycopene stock solution which was prepared every 2 weeks.

A mixed working standard, *i.e.* retinol 500 μg/l (1.75 μmol/l), α-tocopherol 12 mg/l (27.86 μmol/l), lutein 170 μg/l (0.30 μmol/l), lycopene 160 μg/l (0.30 μmol/l), α-carotene 50 μg/l (0.09 μmol/l) and β-carotene 200 μg/l (0.37 μmol/l) was used to monitor sensitivity daily. Retinyl acetate was used as internal standard (850 μg/l) (2.59 μmol/l) and was prepared weekly from the stock solutions.

2.4. Chromatography

The LC system consisted of a Perkin-Elmer pump (Norwalk, CT, USA), a Waters Model 710B autosampler (Milford, MA, USA), a Spectra UV 2000 programmable and variable wavelength UV-Vis detector and an SP 4400 integrator (Spectra-Physics, San Jose, CA, USA). The UV-Vis instrument can perform an automatic zero of the baseline at the end of each sample. The mobile phase (acetonitrile–tetrahydrofuran–methanol, 68:22:7, v/v/v adjusted to 100 v/v with 1% ammonium acetate) was filtered (glass microfibre filters, Whatman, Maidstone, Kent, UK) and pumped at 1.5 ml/min. The column (250 × 4.6 mm I.D.), packed with Nucleosil 100-5 C_{18} 5-μm particles, was obtained from Macherey-Nagel AG (Oensingen, Switzerland). A guard column 15 × 3.2 mm I.D. (Brownlee, Santa Clara, CA, USA) packed with Lichrosorb RP-18 7-μm, preceded the analytical column. The detector was programmed to monitor at 325 nm from 0 to 3.0 min, at 450 nm from 3.0 to 4.9 min, at 290 nm from 4.9 to 7.4 min, at 470 nm from 7.4 to 12.0 min and at 450

nm from 12.0 to 15.0 min, then back to 325 nm at the end of the run. Three minutes after injection, the attenuation was decreased twofold. Fig. 1 shows a chromatogram of mobile phase sample injection with wavelength changes at 3.0, 4.9, 7.4 and 12.0 min.

2.5. Sample preparation

The extraction of plasma was carried out in a room protected from direct sunlight. Plasma or serum (200 μl) was mixed with 200 μl of ethanol containing 850 μg/l (2.59 μmol/l) of retinyl acetate for 1 min. One ml of *n*-hexane, containing 0.4 g of BHT per liter was then added. The tubes were shaken for 10 min on a mechanical shaker, and centrifuged (2000 *g*, 5 min, 20°C) to separate the phases. An 800-μl aliquot of the clear supernatant was removed, evaporated under nitrogen at 40°C and the residue reconstituted in 100 μl of mobile phase without aqueous solvent. A 15-μl portion of this solution was injected onto the HPLC system.

2.6. Recovery

Analytical recovery was measured as described above. Ethanol (0.2 ml) containing internal standard was pipetted into 18 extraction tubes (groups A, B and C). Groups A and B contained 200 μl of mixed working standard that had been previously evaporated to dryness. A 200-μl volume of water was added to each tube of group A and 200 μl of serum to each tube of groups B and C. After mixing, 1 ml of *n*-hexane–BHT was added. After shaking for 10 min on a mechanical shaker the tubes were centrifuged (2000 *g*, 5 min, 20°C) to separate phases. An 800-μl volume of the clear supernatant was removed, evaporated under nitrogen at 40°C and the residue reconstituted in 100 μl of mobile phase without aqueous solvent. A 15-μl portion of this solution was injected onto the HPLC system.

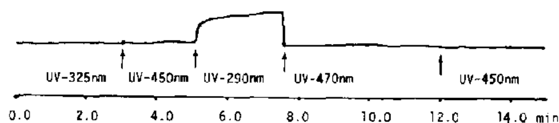

Fig. 1. Chromatogram of a solvent blank obtained by injecting the mobile phase. For chromatographic conditions and mobile phase see Experimental. Detection: UV 325 nm (retinol and retinyl acetate), 450 nm (lutein), 290 nm (tocopherols), 470 nm (lycopene), 450 nm (carotenes).

3. Results

3.1. Quantitation

Different concentrations of standards were prepared from the stock solutions after removing solvent by evaporation under nitrogen and were diluted with HPLC mobile phase without aqueous solution. Linear responses with correlation coefficients of the corresponding regression lines (in parentheses) were observed from 100 to 4300 μg/l (0.35 to 15.00 μmol/l) for retinol (0.99979, $n = 11$), from 3 to 30 mg/l (6.97 to 69.66 μmol/l) for α-tocopherol (0.99996, $n = 6$), from 70 to 1500 μg/l (0.12 to 2.64 μmol/l) for lutein (0.99950, $n = 6$), from 200 to 2000 μg/l (0.37 to 3.73 μmol/l) for lycopene (0.99962, $n = 6$), from 40 to 2000 μg/l (0.07 to 3.73 μmol/l) for α-carotene (0.99999, $n = 5$) and from 40 to 5000 μg/l (0.07 to 9.31 μmol/l) for β-carotene (0.99996, $n = 6$). The lower detection limits were for retinol 10 μg/l (0.03 μmol/l), for α-tocopherol 200 μg/l (0.46 μmol/l), for lutein 5 μg/l (0.01 μmol/l), for lycopene 10 μg/l (0.02 μmol/l), for α-carotene 20 μg/l (0.04 μmol/l), and for β-carotene 20 μg/l (0.04 μmol/l).

3.2. Recovery

The mean recoveries (in percent) for six independent experiments were: retinol 100.1%, α-tocopherol 96.0%, lutein 98.9%, *trans*-lycopene 99.2%, α-carotene 95.5%, β-carotene 101.0% (Table 1).

3.3. Reproducibility of the results

Reproducibility of the method was checked by multiple determination of a pooled human plasma on the same day (the inter-batch precision). The following C.V.s were obtained: retinol 2.5%

Table 1
Accuracy of retinol, α-tocopherol, lutein, lycopene, α- and β-carotene

Compound	Concentration (mean ± S.D., n = 6)(μmol/l)			Recovery[b] (%)
	A[a]	B[a]	C[a]	
Retinol	1.53 ± 0.01	1.73 ± 0.04	3.26 ± 0.09	100.1
Tocopherol	25.54 ± 0.28	22.24 ± 0.48	45.87 ± 0.86	96.0
Lutein	0.30 ± 0.01	0.27 ± 0.01	0.56 ± 0.02	98.9
Lycopene	0.30 ± 0.02	0.48 ± 0.03	0.77 ± 0.02	99.2
α-Carotene	0.153 ± 0.005	0.124 ± 0.006	0.265 ± 0.007	95.5
β-Carotene	0.385 ± 0.016	0.582 ± 0.027	0.977 ± 0.036	101.0

[a] A = water + working standard; B = serum only; C = serum + working standards.
[b] Recovery = $C \times 100/A + B$.

(n = 20), α-tocopherol 2.2% (n = 20), lutein 2.4% (n = 20), *trans*-lycopene 5.8% (n = 20), α-carotene 5.1% (n = 20), β-carotene 4.7% (n = 20). Different samples of the same pooled human plasma were used for each assay over a 12-week period on thirty different days to test day-to-day reproducibility of the method. The intra-batch C.V.s were: retinol 5.7% (n = 30), α-tocopherol 3.9% (n = 30), lutein 5.0% (n = 30), *trans*-lycopene 10.9% (n = 30), α-carotene 11.3% (n = 30), and β-carotene 10.5% (n = 30).

4. Discussion

Fig. 2 shows a chromatogram of a mixed working standard with retinyl acetate as internal standard using the programmable and variable wavelength UV-Vis detector. The change of wavelengths resulted in changes of the baseline at 4.9 and 7.4. The change of baseline at 3.0 was shielded by the programmed change in attenuation at the same time. There was no alteration of baseline at 12.0 min. A typical chromatogram of human plasma is shown in Fig. 3. There was good resolution between retinol, retinyl acetate (internal standard), lutein and zeaxanthin. Tocopherol and carotene isomers were also well resolved. The α-tocopherol was the prevalent isomer form found in our human plasma samples. For the determination of β-cryptoxanthin which was not well resolved from the tocopherol isomers, the use of another visible detector was necessary. We have chosen the internal standard method for calculation in order to obtain better reproducibility. For this purpose retinyl acetate was selected. In our HPLC system retinyl acetate was well resolved from retinol and has also a relatively high absorption coefficient.

This method has been used for almost two years. Over thousand human plasmas from Swiss hospitals and clinics were analyzed as part of a

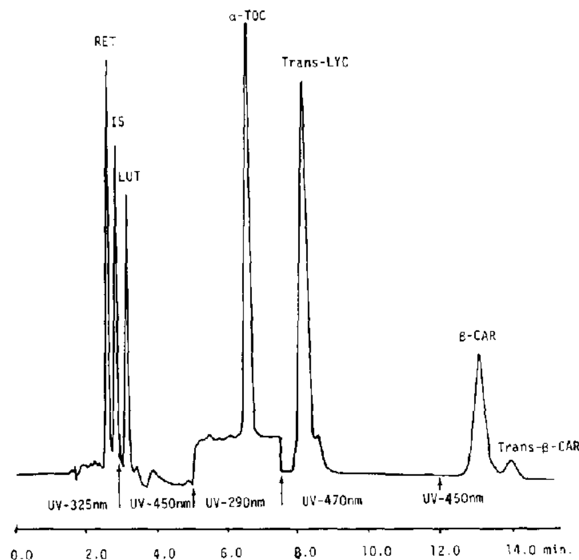

Fig. 2. Chromatogram of a mixed working standard. For chromatographic conditions and mobile phase, see Experimental. Legend: RET = retinol, I.S. = retinyl acetate (internal standard), LUT = lutein, ZEA = zeaxanthin, α-TOC = α-tocopherol, LYC = lycopene, β-CAR = β-carotene and *trans*-β-CAR = *trans*-β-carotene.

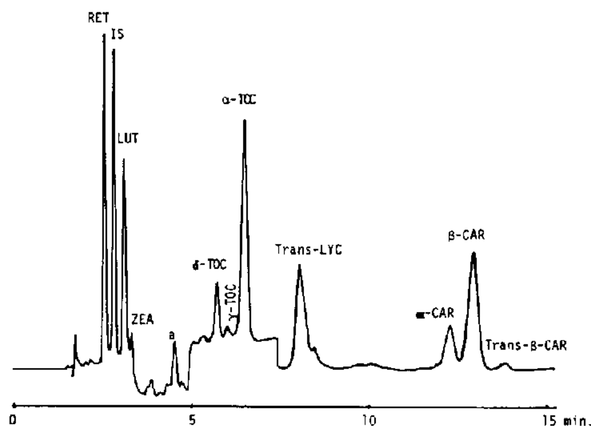

Fig. 3. Chromatogram of a human serum sample. For chromatographic conditions and mobile phase, see Experimental. Legend: RET = retinol, I.S. = retinyl acetate (internal standard), LUT = lutein, ZEA = zeaxanthin, δ-TOC = δ-tocopherol, γ-TOC = γ-tocopherol, α-TOC = α-tocopherol, LYC = lycopene, α-CAR = α-carotene, β-CAR- = β-carotene and *trans*-β-CAR = *trans*-β-carotene; a = unidentified carotenoid.

EURAMIC study of the European Community. During this period the pre-column was changed three times and the analytical columns changed twice when resolution between retinol and retinyl acetate was less than 1.

The concentrations measured during this period ranged from 60 to 750 μg/l (0.20–2.62 μmol/l) for retinol, 1 to 38 μg/l (2.32–8.82 μmol/l) for α-tocopherol, 10 to 800 μg/l (0.02–1.41 μmol/l) for lutein, 60 to 540 μg/l (0.11–1.01 μmol/l) for *trans*-lycopene, not detectable to 500 μg/l (0.93 μmol/l) for α-carotene, and 20 to 1500 μg/l (0.04–2.79 μmol/l) for β-carotene. Sample handling is relatively quick and simple. The method is suitable for routine analysis and can be performed automatically using a single detector. It is also inexpensive because the mobile phase can be reused many times without altering the retention time of the compounds. We have observed no interfering compound, as shown in Fig. 1, obtained by injecting 15 μl of the mobile phase. The HPLC system has proven to be extremely stable giving reproducible and reliable results. Temperature control of the automatic injector is suggested if laboratory temperature greatly varies.

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