Hindawi Publishing Corporation
Journal of Chemistry
Volume 2013, Article ID 460242, 7 pages
http://dx.doi.org/10.1155/2013/460242

Research Article

Determination of Retinol, α-Tocopherol, Lycopene, and β-Carotene in Human Plasma Using HPLC with UV-Vis Detection: Application to a Clinical Study

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Received 26 June 2012; Revised 13 August 2012; Accepted 29 August 2012

Academic Editor: Gerald Culioli

A method is described here for the simultaneous determination of retinol, α-tocopherol, lycopene, and β-carotene in human plasma. The effectiveness of various protein precipitants and extraction solvents was tested. After adequate sample preparation, the samples were injected directly into the HPLC system. The separation was realized on an analytical reversed-phase column with a UV-Vis detection. The analytical performance of this method was satisfactory. The intraassay and interassay coefficients of variation were below 10%. The recoveries were as follows: 97.0% (CV 2.4%) for retinol, 94.6% (CV 1.7%) for α-tocopherol, 91.9% (CV 3.6%) for lycopene, and 93.9% (CV 4.2%) for β-carotene. The levels of selected fat-soluble vitamins in plasma of patients with cardiovascular disease were measured and discussed.

1. Introduction

Fat-soluble vitamins such as α-tocopherol, β-carotene, and lycopene are very important antioxidants [1–3]. Reduced levels of these antioxidants in plasma have been reported to be associated with numerous diseases [3–6]. Association between high plasma levels of fat-soluble antioxidant vitamins and lower risk of atherosclerosis was described in several studies [6]. Vitamin E is constituted by a group of eight isomers that include both tocopherols and tocotrienols. α-Tocopherol is the most biologically active form of vitamin E [7]. Lycopene is a powerful quencher of singlet oxygen that produced during exposure to ultraviolet light [8, 9]. β-Carotene, the main dietary source of Vitamin A, is also a quencher of singlet oxygen [10, 11]. Numerous methods have been described for the analysis of fat-soluble antioxidant vitamins in various biological matrices. Up to date, several reversed-phase high-performance liquid chromatographic (RP-HPLC) methods using C_{18} or C_{30} stationary phase coupled with spectrophotometric, fluorometric, electrochemical, or mass spectrometric detection were developed [1, 2, 12–16]. The instability of fat-soluble antioxidant vitamins during sample storage and preparation is the major problem at their measurement. Therefore, sample preparation is essential for accurate analysis of such compounds. While α-tocopherol together with retinol are relative stable, β-carotene and lycopene are easily subject to degradation. Degradation of lycopene and β-carotene is influenced by temperature, light, and dissolved oxygen [17–19].

The aim of this study was to develop a reliable RP-HPLC method for the measurement of fat-soluble antioxidant vitamins in human plasma and to prevent their degradation during sample preparation.

2. Experimental

2.1. Reagents and Chemicals. Retinol, α-tocopherol, β-carotene, lycopene, retinyl acetate, α-tocopheryl acetate, β-apo-8′-carotenal, retinyl palmitate, and 2,6-di-*tert*-butyl-4-methylphenol (butylated hydroxytoluene) were obtained from Sigma (St. Louis, MO, USA). HPLC gradient grade methanol, ethanol, 1-propanol, 2-propanol, acetonitrile, and n-hexane were obtained from Merck (Darmstadt, Germany). All the other chemicals were of analytical grade.

2.2. Standards Preparation. Stock solutions of retinol (≈20 mg/L; 105 μmol/L), α-tocopherol (≈1 g/L; 2322 μmol/L), retinyl acetate (≈20 mg/L; 70 μmol/L), and α-tocopheryl acetate (≈1 g/L; 2115 μmol/L) were prepared in ethanol. Stock solutions of β-carotene (≈20 mg/L; 37 μmol/L), lycopene (≈20 mg/L; 37 μmol/L), retinyl palmitate (≈20 mg/L; 38 μmol/L), and β-apo-8′-carotenal (≈20 mg/L; 48 μmol/L) were prepared in n-hexane. All stock solutions were stored at −20°C (maximum of three months). Work solutions of individual vitamins and internal standards were prepared daily and the concentrations were verified spectrophotometrically using molar absorptions:

concentration of retinol = A/53 000 (mol/L) at 325 nm [20];

concentration of α-tocopherol = A ∗ 248.1 (μmol/L) at 292 nm [21];

concentration of β-carotene = A ∗ 4.74 (μmol/L) at 450 nm [22];

concentration of lycopene = A ∗ 3.56 (μmol/L) at 468 nm [22];

concentration of retinyl acetate = A/49602 (mol/L) at 325 nm [23];

concentration of α-tocopheryl acetate = A/1891 (mol/L) at 290 nm [24].

2.3. Instrumentation. Chromatographic analysis was performed with a liquid chromatograph (Ecom, Prague, Czech Republic) equipped with a LCP 4100 solvent delivery system, an AS 54 autosampler, a LCO 101 column oven, and a LCD 2084 variable wavelength spectrophotometric detector. Data were collected digitally using Clarity chromatography software (DataApex, Prague, Czech Republic). Spectrophotometric analyses were carried out on a Shimadzu (Kyoto, Japan) UV-1700 PharmaSpec spectrophotometer.

2.4. Subjects. A total of 129 patients that diagnosed with nonacute coronary angiography for chest pain (40 women in the age 61 ± 7 years and 89 men in the age 59 ± 7 years) were included in this study. All patients gave written informed consent to participate in this research study, which was approved by the Hospital Committee on Human Research (Regional Hospital of Pardubice, Czech Republic, protocol 336/2010) according to the Helsinki Declaration.

2.5. Patients' Inclusion and Exclusion Criteria. Criteria for the submission into the study were (1) clinically significant coronary stenosis without subsequent percutaneous coronary intervention (PCI) treatment (coronary stenosis of >50% of the left main coronary artery or >70% of the epicardial coronary arteries without PCI treatment); (2) stenosis with PCI treatment (stenosis of the same grade with subsequent PCI treatment); (3) patients without any stenosis (symptoms imitating coronary artery disease with normal angiographic finding and no stenosis). All participants underwent angiography and completed a questionnaire. Patients who had any serious health complications and patients with increased high-sensitivity C-reactive protein levels (>10 mg/L) were excluded. None of the studied subjects exhibited renal, hepatic, gastrointestinal, pulmonary, or oncological diseases.

2.6. Blood Samples Collection. Venous blood samples were obtained under standard conditions, from 7 to 8 a.m. after fasting for at least 12 hours the day after coronarographic examination. Blood was collected into tubes with EDTA (the Vacuette Detection Tube, No. 454246, Greiner Labortechnik Co., Kremsmünster, Austria) that covered with an aluminum foil to minimize exposure of blood samples to daylight. Plasma was separated from blood cells by centrifugation (1700 ×g, 15 min, 8°C) and immediately stored at −80°C in 1.5-mL amber polypropylene tubes.

2.7. Sample Preparation. Retinyl acetate and α-tocopheryl acetate were used as internal standards. They are not present in human plasma and their chromatographic properties are quite similar. On the other hand, retinyl palmitate and β-apo-8′-carotenal were detected in most of the samples.

All procedures were performed in a darkened room.

For the analysis of blood plasma, 10 μL of the internal standard of retinyl acetate solution in ethanol (≈40 μmol/L; 13 μg/mL) and 10 μL of the internal standard α-tocopheryl acetate solution in ethanol (≈2 mmol/L; 945 μg/mL) were pipetted into a well-capped 1.5-mL amber polypropylene tube. 200 μL of plasma were added and the content mixed vigorously on a vortex mixer for 10 min. 200 μL of cold ethanol were added and the solution was vortexed for 60 s. 500 μL of cold n-hexane containing butylated hydroxytoluene (1 g/L) were then added, the solution was vortexed for 5 min, and centrifuged (22 000 ×g, 5 min, 4°C). The upper hexane layer was transferred into 5-mL amber glass tubes. The hexane extraction process was repeated two times. Combined hexane extracts were evaporated to dryness, under nitrogen, at 4°C (Linde Gas, Prague, Czech Republic). The dried residue was resuspended in 200 μL of cold ethanol and vortexed for 60 s. The sample was then filtered through a nylon filter (pore size 0.20 μm, 4 mm diameter, Supelco, Bellefonte, PA, USA), transferred into 0.2-mL cramped amber vial, and purged with pure nitrogen for 10 s.

The stock solutions of fat-soluble vitamins were diluted with ethanol (retinol, α-tocopherol) or n-hexane (lycopene, β-carotene) to give a series of mixed working standards. To 200 μL of mixed solution of standards, 10 μL of the internal standard of retinyl acetate solution in ethanol (≈40 μmol/L; 13 μg/mL), 10 μL of the internal standard α-tocopheryl acetate solution in ethanol (≈2 mmol/L; 945 μg/mL), 200 μL of deionized water, and 500 μL of cold n-hexane containing butylated hydroxytoluene (1 g/L) were carefully added. Prepared standards were subjected to the same procedure as described above for plasma samples.

For recovery experiments, 10 μL of mixed solution of individual vitamins at different concentrations was added to 190 μL of plasma. The next steps were the same as for plasma sample preparation.

2.8. Chromatographic Analysis. The chromatographic analysis of selected fat-soluble vitamins was accomplished using an isocratic elution on a Discovery HS C_{18}, 150 × 4 mm i.d., 5 μm analytical column that fitted with a Discovery C_{18}, 20 × 4 mm i.d., 5 μm guard column (Supelco, Bellefonte, PA, USA) at 40°C. The mobile phase was a mixture of methanol and ethanol (75 : 25, v/v). The flow rate was kept constant at 0.8 mL/min. Optimum response of α-tocopherol and α-tocopheryl acetate was observed when wavelength was set at 292 nm, while retinol together with retinyl acetate were monitored at 325 nm, β-carotene at 450 nm, and lycopene at 468 nm. The amount of individual vitamins was quantified from the corresponding peak area ratio of vitamin/internal standard (α-tocopherol/α-tocopheryl acetate; retinol, lycopene, and β-carotene/retinyl acetate) using Clarity chromatography software (DataApex). The concentration of each analyte in the samples was determined from its calibration curve.

2.9. Statistical Analysis. The data are presented as mean ± S.D. Differences between studied groups were analyzed using the Student's t-test. The correlation analysis was carried out using Spearman Rank Order Correlation, and regression analysis was carried out using the least squares method (software QCexpert, Trilobyte, Pardubice, Czech Republic). A $P < 0.05$ value was considered statistically significant.

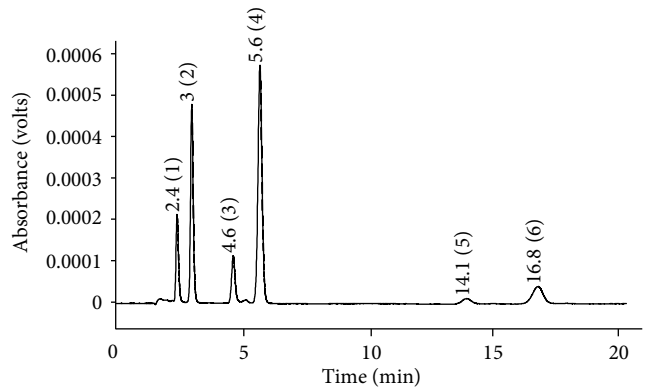

Figure 1: An HPLC chromatogram of retinol (1.00 μmol/L; 0.29 μg/mL), α-tocopherol (15.0 μmol/L; 6.46 μg/mL), lycopene (0.020 μmol/L; 0.011 μg/mL), and β-carotene (0.100 μmol/L; 0.054 μg/mL) in standard solution. Peaks: (1) retinol, (2) retinyl acetate, (3) α-tocopherol, (4) α-tocopheryl acetate, (5) lycopene, and (6) β-carotene. HPLC conditions: an isocratic elution (mobile phase : methanol-ethanol, 75 : 25, v/v); the stationary phase was an analytical column Discovery HS C_{18}, 150 × 4 mm i.d., 5 μm fitted with a Discovery C_{18}, 20 × 4 mm i.d., 5 μm guard column; the flow rate was kept constant at 0.8 mL/min, separation ran at 40°C. Retinol together with retinyl acetate was monitored at 325 nm, α-tocopherol together with α-tocopheryl acetate was monitored at 292 nm, lycopene was monitored at 468 nm, and β-carotene was monitored at 450 nm.

3. Results and Discussion

3.1. Sample Preparation, Stability. Sample preparation is essential for accurate analysis. The stability of selected fat-soluble vitamins in human plasma during the sample treatment was investigated. Several protein precipitants were tested. Protein precipitant of the different temperature (methanol, acetonitrile, ethanol, 1-propanol, and 2-propanol) was carefully added to plasma. Then n-hexane containing or not an antioxidant (butylated hydroxytoluene) was carefully added. Fat-soluble vitamins and internal standards in plasma samples were found to be stable at 4°C for at least 8 h under these conditions: a protein precipitation with ethanol, cooled to −20°C; extraction with n-hexane containing butylated hydroxytoluene, cooled to −20°C; centrifugation at 4°C; evaporation to dryness, under pure nitrogen, at 4°C; resuspension of a residue in ethanol, cooled to −20°C. It is very important that extracts were to be dried as quickly as possible. Fat-soluble vitamins, especially lycopene, are not stable in n-hexane. Fat-soluble vitamins in human plasma samples that stored at −80°C are stable for at least one year. It is necessary that all procedures were to be performed in a darkened room.

3.2. High-Performance Liquid Chromatographic Assay of Fat-Soluble Vitamins. Fat-soluble vitamins were separated on a reversed-phase column using an isocratic system of methanol and ethanol. The mobile phase was optimized in order to obtain the best separation of the analytes in the shortest time. Standard solutions of fat-soluble vitamins and internal standards as well as pooled plasma samples were used for studying the mobile phase composition. Several eluents (mixtures of organic solvents such as acetonitrile, methanol, ethanol, 1-propanol, 2-propanol, and n-hexane) and several gradients were assessed. The best results were obtained for the conditions that described in "Chromatographic Analysis." Column temperature was changed from 25 to 45°C. Optimal temperature interval was from 40 to 45°C. The criteria were resolution, stability of the absorbance, and analysis duration. According to our results, we can conclude that the presented method is highly robust. HPLC chromatograms of fat-soluble vitamins in standard solution and human plasma are shown in Figures 1 and 2. The precision of fat-soluble vitamin analysis in plasma samples is shown in Table 1. To determine the same-day precision, the plasma samples were analyzed ten times in the same day under the same conditions. Similarly, data on the between-day precision were obtained using the same plasma samples, analyzed on ten different days. The coefficients of variation were below 10%. The spike recoveries ranged between 93.8 and 99.4% for retinol, 92.2–95.9% for α-tocopherol, 87.7–95.8% for lycopene, and 88.6–98.8% for β-carotene (Figure 3). Calibration curves (9-point for determining analytical parameters and 7-point for routine analysis) were linear over the whole tested range (Figure 4). The calibration curve parameters obtained as an average from ten standard curves are shown in Table 2. The lowest concentration that could be quantified with acceptable accuracy and precision was 0.1 μmol/L (2.2 pmol/inject) for retinol, 2.1 μmol/L (46.2 pmol/inject) for α-tocopherol, 0.020 μmol/L (0.44 pmol/inject) for lycopene, and 0.021 μmol/L

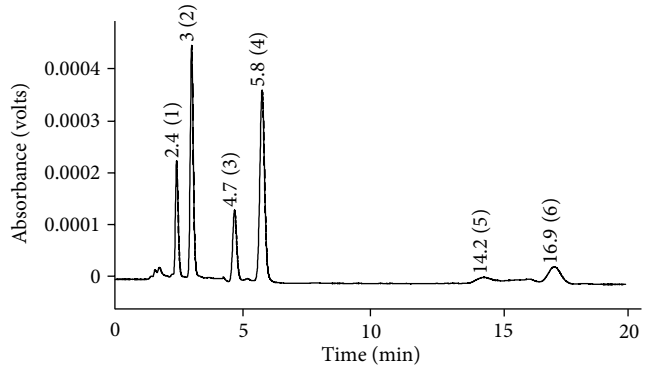

Figure 2: An HPLC chromatogram of retinol (1.14 μmol/L; 0.33 μg/mL), α-tocopherol (27.3 μmol/L; 11.76 μg/mL), lycopene (0.021 μmol/L; 0.011 μg/mL), and β-carotene (0.073 μmol/L; 0.039 μg/mL) in human plasma. Peaks: (1) retinol, (2) retinyl acetate, (3) α-tocopherol, (4) α-tocopheryl acetate, (5) lycopene, and (6) β-carotene. HPLC conditions: see Figure 1.

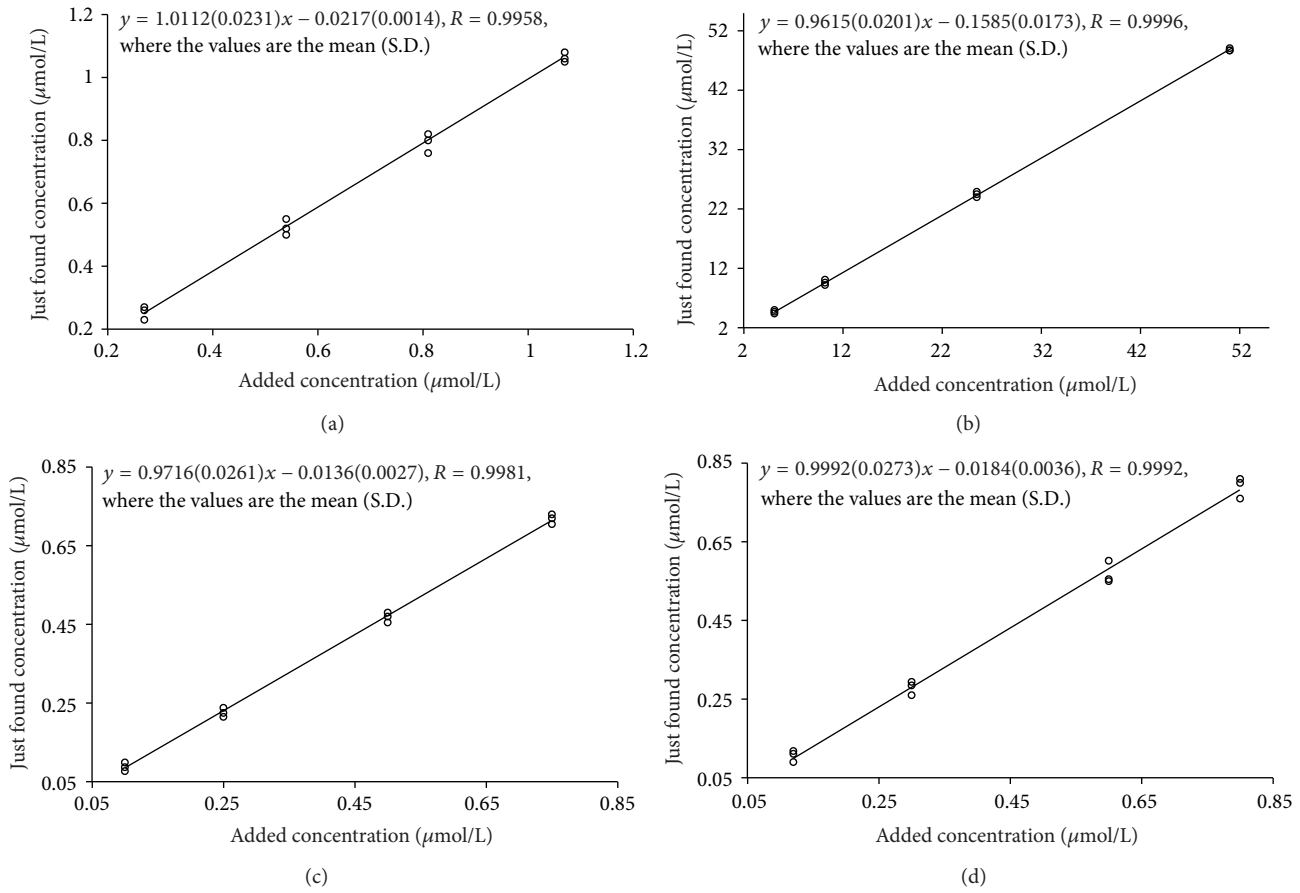

Figure 3: Recovery experiment: retinol (a), α-tocopherol (b), lycopene (c), and β-carotene (d). Values of triplicate assays are recorded. Slopes correspond to the mean recovery 97.0% (S.D. = 2.4%) for retinol, 94.6% (S.D. = 1.7%) for α-tocopherol, 91.9% (S.D. 3.6%) for lycopene, and 93.9% (S.D. = 4.2%) for β-carotene.

(0.46 pmol/inject) for β-carotene. Furthermore, the limits of detection for retinol, α-tocopherol, lycopene, and β-carotene, defined as a signal-to-noise (S/N) ratio of 3 : 1, were 0.03 μmol/L (0.7 pmol/inject), 0.63 μmol/L (13.9 pmol/inject), 0.006 μmol/L (0.13 pmol/inject), and 0.006 μmol/L (0.13 pmol/inject), respectively.

3.3. Determination of Selected Fat-Soluble Vitamins in Human Plasma of Patients with Cardiovascular Diseases. The role of retinol, α-tocopherol, and carotenoids in both physiological and pathological processes has been widely discussed. Many of the biological actions of these fat-soluble vitamins have been attributed to their antioxidant properties. Several

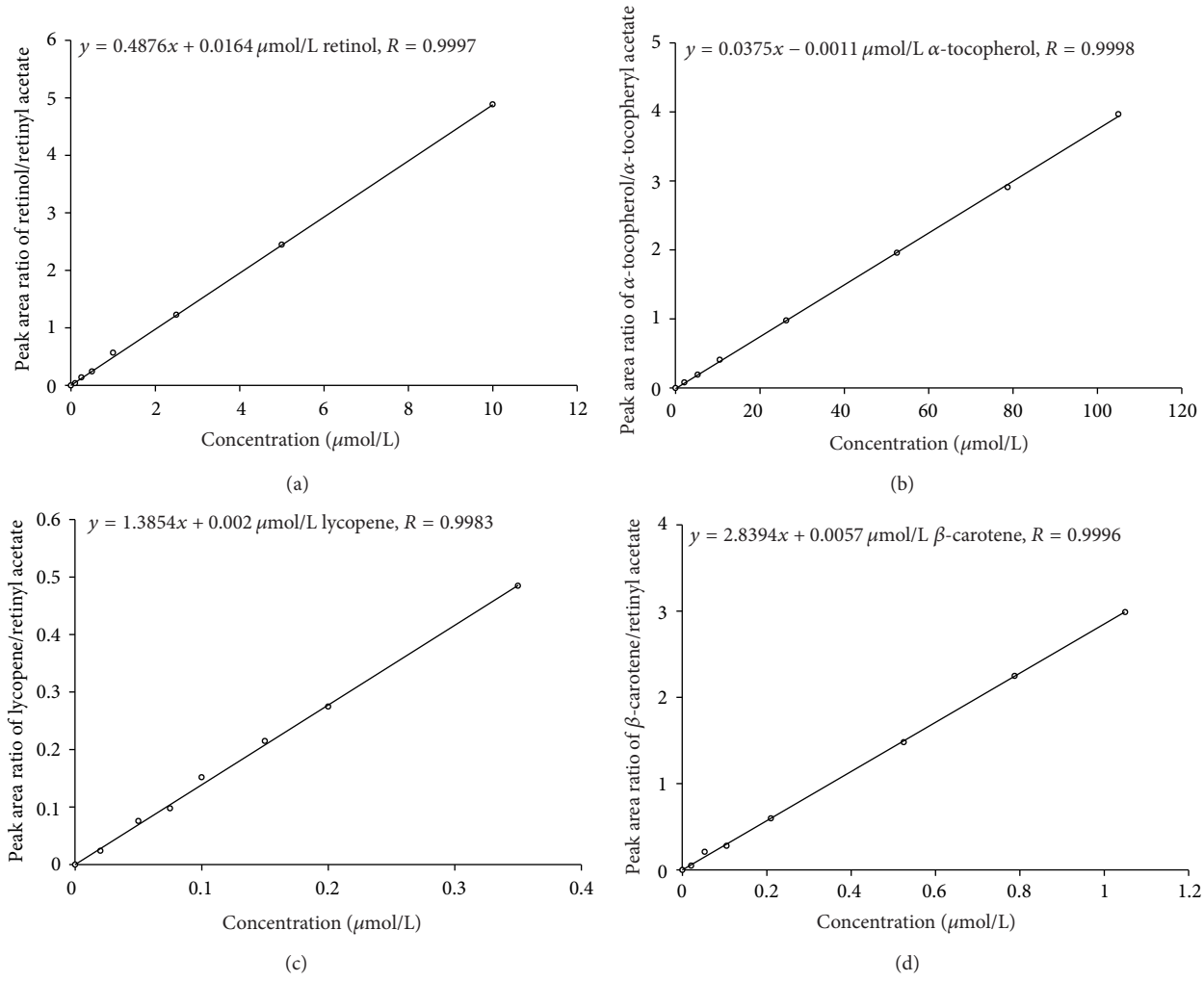

Figure 4: Typical calibration curves for HPLC quantification of retinol (a), α-tocopherol (b), lycopene (c), and β-carotene (d).

Table 1: Precision of retinol, α-tocopherol, lycopene, and β-carotene in human plasma.

	Retinol mean ± S.D., μmol/L	CV, %	α-Tocopherol mean ± S.D., μmol/L	CV, %	Lycopene mean ± S.D., μmol/L	CV, %	β-Carotene mean ± S.D., μmol/L	CV, %
(A) Precision (within day)								
10	1.27 ± 0.07	5.5	22.1 ± 0.69	3.1	0.069 ± 0.005	7.2	0.270 ± 0.018	6.7
10	3.02 ± 0.15	5.0	28.2 ± 0.73	2.6	0.097 ± 0.006	6.2	0.615 ± 0.029	4.7
(B) Precision (between day)								
10	3.05 ± 0.21	6.9	28.8 ± 1.21	4.2	0.099 ± 0.009	9.1	0.611 ± 0.048	7.9

Table 2: Average parameters of 10 calibration curves for the HPLC method.

Standard	Regression equation	Mean slope 95% confidence interval	Intercept (μmol/L)[2] 95% confidence interval	Correlation coefficient
Retinol[1]	$y = 0.4876x + 0.0164$	0.4876 (0.4528–0.5221)	−0.03 (−0.06–0.01)	0.9997
α-Tocopherol[1]	$y = 0.0375x - 0.0011$	0.0375 (0.0342–0.0402)	0.03 (−0.21–0.26)	0.9998
Lycopene[1]	$y = 1.3854x + 0.0020$	1.3854 (1.2731–1.4001)	−0.001 (−0.005–0.003)	0.9983
β-Carotene[1]	$y = 2.8394x + 0.0057$	2.8394 (2.5978–3.0826)	−0.002 (−0.007–0.002)	0.9996

[1]Nine-point for the determination of analytical parameters and seven-point for routine analysis. [2]The x-intercept (in μmol/L) is the point at which the line crosses the x-axis (where the y value equals 0).

Table 3: Comparison of levels of selected fat-soluble antioxidant vitamins between the patients and a control group.

	PCI (1; $n = 45$)	P (2; $n = 37$)	N (3; $n = 47$)	1 versus 3	2 versus 3
Age (years)	59 ± 5	61 ± 6	57 ± 6	—	—
Female/male	9/36	6/31	25/22	—	—
Retinol (μmol/L; μg/mL)	1.21 (0.49); 0.35 (0.14)	1.26 (0.33); 0.36 (0.09)	1.14 (0.32); 0.33 (0.09)	—	—
α-Tocopherol (μmol/L; μg/mL)	22.1 (4.4); 9.52 (1.90)	23.6 (9.7); 10.16 (4.18)	23.7 (5.7); 10.21 (2.46)	—	—
Lycopene (μmol/L; μg/mL)	0.025 (0.010); 0.013 (0.005)	0.038 (0.023); 0.020 (0.012)	0.039 (0.009); 0.021 (0.005)	*	—
β-Carotene (μmol/L; μg/mL)	0.095 (0.117); 0.051 (0.063)	0.106 (0.120); 0.057 (0.064)	0.139 (0.098); 0.075 (0.053)	***	**

Results are expressed as the mean value with the estimated standard deviation (S.D.), the statistical significance of a difference: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (Student's t-test).
PCI: patients with stenosis and with subsequent percutaneous coronary intervention.
P: patients with stenosis without subsequent percutaneous coronary intervention.
N: patients without any stenosis.

studies showed an inverse association between fat-soluble antioxidant vitamins in plasma levels and atherosclerotic progression in arteries [25, 26]. The patients with cardiovascular disease were divided into 3 groups according to the results of angiographic examination. Blood samples were collected the day after coronarographic examination. Mean values (±S.D.) of selected fat-soluble vitamins are shown in Table 3. In patients with stenosis and with subsequent percutaneous coronary intervention (PCI), significantly more in men, lower levels of β-carotene and lycopene were detected. In patients with stenosis without subsequent percutaneous coronary intervention (P), significantly more in men, lower levels of β-carotene were detected. Now our results revealed a significant correlation of coronary artery disease occurrence and low carotenoids levels. Beside antioxidant properties, carotenoids have other important functions such as protection of DNA against peroxidation, enhancement of gap junctional communication, immunomodulation, and tumor-suppressive activity. However, the exact mechanisms leading to the beneficial effects in atherosclerosis are still under debate. Levels of β-carotene and lycopene were markedly below the recommended plasma concentrations in all groups. Women generally have higher concentrations of fat-soluble antioxidant vitamins, especially carotenoids. Several factors influence plasma concentrations of such compounds: sex, age, dietary intake, smoking and drinking habits, and seasonality [27–29]. Significant correlation between retinol concentration and age ($R = -0.480$, $P = 0.0098$) in patients without any stenosis (N) and between lycopene concentration and age ($R = -0743$, $P = 0.0108$) in P patients was observed.

4. Conclusions

A reliable, selective, and sensitive HPLC method with spectrophotometric detection for the determination of retinol, α-tocopherol, lycopene, and β-carotene in human plasma was developed. Proper sample preparation preventing fat-soluble antioxidant vitamins degradation is required to achieve accurate analyses. The crucial points are sample collection, protein precipitation, and liquid-liquid extraction. The best results were obtained under these conditions:

tubes for blood sampling containing EDTA and covered with aluminum foil;

fast freezing of plasma samples (−80°C);

cold ethanol (−20°C) as a protein precipitant;

extraction with cold n-hexane (−20°C) containing an antioxidant (butylated hydroxytoluene);

evaporation to dryness, under pure nitrogen (purity 4.6 and more), at 4°C;

resuspension of the obtained residue in cold ethanol (−20°C);

transfer of the prepared samples into 0.2-mL cramped amber vials and treatment with pure nitrogen for 10 s.

Acknowledgment

This work was supported by Grants SGFChT07/2012 and MSMT 0021627502 from the Czech Ministry of Education.

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