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Liquid chromatographic method for the simultaneous determination of different lipid-soluble antioxidants in human plasma and low-density lipoproteins

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Abstract

We describe a reverse phase HPLC method, employing a simple methanol:water gradient as mobile phase, for the determination of several lipophilic antioxidants, such as retinol, γ-tocopherol, α-tocopherol, lycopene, α-carotene and β-carotene among others, using UV detection. Additionally, this method allows the simultaneous separation of probucol, an hypocholesterolemic drug with antioxidant properties. Retinol acetate and α-tocopherol acetate were added to samples as internal standards. A NovaPack ODS C18, 150 × 3.9 mm, 0.4 μm column was used and the flow rate was set constant at 1 m/min, which allowed the separation of all the desired antioxidants in a total run time of 35 min. A photodiode array detector was used because of its advantages to study the purity of the peaks, however, any programmable multiwavelength UV/VIS detector could be employed given the good resolution of the peaks. The analytical recoveries of the studied compounds were >96% and the detection limits were: retinol 0.050 μg/ml, γ-tocopherol 0.137 μg/ml, α-tocopherol 0.906 μg/ml, lycopene 0.022 μg/ml, α-carotene 0.008 μg/ml, β-carotene 0.015 μg/ml and probucol 1.503 μg/ml. The intra- and inter-assay coefficients of variation were calculated by using two human plasma samples with different levels of lipophilic antioxidants. The simplicity, rapidity and economy, make this method suitable for the routine measurement of plasma and low-density lipoproteins antioxidants, and may also be used in large scale epidemiological studies. The method has been used to measure antioxidants in samples from patients undergoing treatment with probucol, showing there is a good correlation between the probucol content in LDL and that in total plasma.
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1. Introduction

Epidemiological studies have consistently shown that high intakes of Vitamin A, Vitamin E and carotenoids are associated with low risk of several chronic diseases, including cardiovascular disease, age-related degeneration and some cancers [1,2]. Thus, these micronutrients exhibit multiple biological actions that may protect against disease.

Oxidation of low-density lipoproteins (LDL) is generally believed to promote atherosclerosis primarily by leading to an increased uptake of oxidized LDL by macrophages and subsequent foam-cell formation [3]. Over the last decade, interest has grown on the possibility that foods rich in antioxidant vitamins may reduce the risk of atherosclerotic disease by protecting LDL from oxidative modification. Supplements of Vitamin E, Vitamin C, β-carotene, flavonoids or a combination of them have been shown to inhibit lipid peroxidation ex vivo [4–6] and epidemiological studies have revealed inverse relationships between the intake of antioxidants and cardiovascular disease [7]. However, the benefit of antioxidant supplements on cardiovascular disease is controversial [8,9]. For example, probucol, a lipid-soluble drug, lowers cholesterol levels and has been shown to reduce progression of atherosclerosis in animal models [10,11], but it is not clear whether this protective mechanism can be attributed or not to its known antioxidant properties.

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Actual measurements of plasma antioxidants in subjects receiving vitamin supplements or probucol are needed in order to evaluate the real antioxidant charge in these subjects. Sensitive methods which allow the simultaneous separation of all of these compounds would ease this task. We report herein the application of a high-pressure liquid chromatography method with visible/ultraviolet detection for the quantitation of retinol, γ-tocopherol, α-tocopherol, lycopene, α-carotene, β-carotene, and probucol in plasma and isolated LDL. The procedure is simple, rapid and inexpensive, which make it suitable for big scale studies.

2. Experimental

2.1. Materials

Solvents used were HPLC-grade quality (Merck) and were degassed before use. Water was nanopure grade (>18 MΩ, Millipore, Bedford, MA). Retinol (all *trans*, crystalline), (+)-α-tocopherol (≈95%, in an oil form), (+)-γ-tocopherol (crystalline), (+)-α-tocopherol acetate (oil form), lycopene (90% to 95%, crystalline form), α-carotene (type V, crystalline and essentially free of β-carotene), and β-carotene (all *trans*, type II, crystalline and essentially free of α-carotene) were purchased from Sigma–Aldrich. Retinol acetate (all *trans*, crystalline) was obtained from Fluka. Probucol was a gift from Marian Merrell Dow, S.A. All compounds were stored under argon at −20 °C.

2.2. Blood withdrawal and LDL isolation

Blood samples were withdrawn from both healthy individuals (controls) and hyperlipidemic patients that were being treated with probucol (1 g daily). Some of the patients were also studied 1 month after probucol treatment was withdrawn. At the time of the examination, after an overnight fast, blood samples were collected into tubes containing Na_2-EDTA (1 mg/ml final concentration). Plasma was then immediately separated by centrifugation at 1200 × *g* for 20 min at 4 °C. Plasma aliquots for subsequent determination of lipid-soluble antioxidants were kept at −80 °C until HPLC analysis. Total cholesterol was measured by an enzymatic colorimetric method (Menarini, Firenze, Italy).

LDL were isolated by single spin ultracentrifugation (VTi 50 rotor, Beckman) [12]. Briefly, a discontinuous salt gradient was produced in ultracentrifuge tubes by layering 10 ml of plasma adjusted to a density of 1.30 g/ml with KBr, underneath a 1.006 g/ml solution containing EDTA-Na_2 1 mmol/l to minimize lipoprotein oxidation. The tubes were placed in a Beckman VTi-50 vertical rotor and centrifuged at 50,000 rpm for 2 h 45 min, at 10 °C. The LDL fraction (approximately 0.5 ml) was aspired into a syringe by piercing the side of the tube with a needle. Purity of LDL was confirmed by agarose gel electrophoresis, showing a single band with β mobility.

2.3. Standard solutions and standard curve preparation

Solutions were prepared in the cold room at 4 °C under indirect light. Individual stock solutions were prepared fresh, except for the Vitamin E homologs, which are stable for 3 months at −20 °C as determined. The retinol (2 mg/ml), γ-tocopherol (0.8 mg/ml), α-tocopherol (10 mg/ml), and probucol (10 mg/ml) standards were prepared in absolute ethanol. Lycopene (0.08 mg/ml), α-carotene (0.04 mg/ml) and β-carotene (0.08 mg/ml) were dissolved in chloroform. Probucol stock standard was prepared gravimetrically, whereas the actual concentrations of the other stock standards were determined by spectrophotometry (Beckman, DU 640), using the appropriate absorption coefficients in the literature [13]. Retinol acetate (0.5 mg/ml) was used as internal standard for retinol and carotenoids while α-tocopherol acetate (0.4 mg/ml) was for Vitamin E homologs, both dissolved in ethanol. These solutions were stored separately at −80 °C. The criterion of stability of the stock standard solutions was based on their spectra of absorbance and on the absence of degradation products as determined by HPLC.

A working combined standard solution in ethanol was prepared by mixing the stock solutions, and serial dilutions from this were made in ethanol. In addition, an internal standard mixture in ethanol was prepared daily, by diluting the respective stock solutions to a final concentration of 5 μg/ml retinol acetate and 40 μg/ml α-tocopherol acetate approximately. Standard curves were prepared with a plasma pool by the method of standard additions; all additions were performed in triplicate. A fixed volume (50 μl) of each diluted working combined standard solution was added to 200 μl of plasma. Then, 50 μl of the mixture of internal standards plus 100 μl of ethanol and 200 μl of methanol were added, and the samples were further processed with hexane for extraction as described below. To calculate the recoveries of the antioxidants of interest, plasma samples were supplemented with known amounts of pure antioxidants and were analyzed simultaneously with the non-supplemented plasma samples, the difference for each antioxidant was compared to the value of the pure standard, dissolved in ethanol, directly injected into the cromatograph.

2.4. Sample extraction

Plasma samples were extracted in duplicates, on ice and with degassed solvents. Fifty microliters of the mixture of internal standards in ethanol were added to 200 μl of plasma, followed by 150 μl ethanol and 200 μl methanol in order to precipitate proteins. Tubes were vortexed vigorously. Then, 1 ml of hexane was added, vortexed for 1 min, and centrifuged at 1200 × *g* at 4 °C for 10 min. The supernatant was transferred to another tube and the extraction was repeated once. Then, the supernatants were combined, frozen with liquid nitrogen, and evaporated to dryness in a centrifugal evaporator (Speed-Vac, Savant Instruments,

Farmingdale, NY). The residue was reconstituted in 200 μl of propanol:acetonitrile (50:50, v/v), vortexed and transferred to autosampler microvials. Fifty microliters of the extract were injected.

2.5. Chromatography conditions

HPLC was performed using a Beckman System "Gold" equipment consisting of a 126 binary pump, a 168 programmable diode array detector, and an automated sample injector (Mod. 123 Gilson). The extract (50 μl) was separated on an ODS C18 column (NovaPack 150 × 3.9 mm, 0.4 μm) using a gradient of methanol/water at a flow rate of 1 ml/min as follows: methanol/water (90:10) was changed linearly over 10 min to 100% methanol, then 100% methanol was continued for 30 min, and finally methanol/water (90:10) again. Column temperature was maintained at 40 °C. To increase stability of the redissolved plasma extracts, the autosampler was kept at 4 °C. Detection was performed simultaneously at multiple wavelengths and absorbances were recorded into two channels as shown in Fig. 1: 244 nm for probucol, 326 nm for retinol and retinol acetate, 292 nm for γ-tocopherol, α-tocopherol and α-tocopherol acetate, and 450 nm for carotenoids. Peak identification and purity were based on the absorption spectrum in the UV or visible regions, retention time and peak spiking with pure standards.

2.6. Assay precision and sample stability

To asses daily and long-term laboratory performance, two control plasma pools were prepared, with different concentrations of retinol, tocopherols and carotenoids, and aliquots were kept frozen at −80 °C until use. The coefficients of variation were calculated by replicating extraction and analysis of these control plasma pools: for the intra-assay

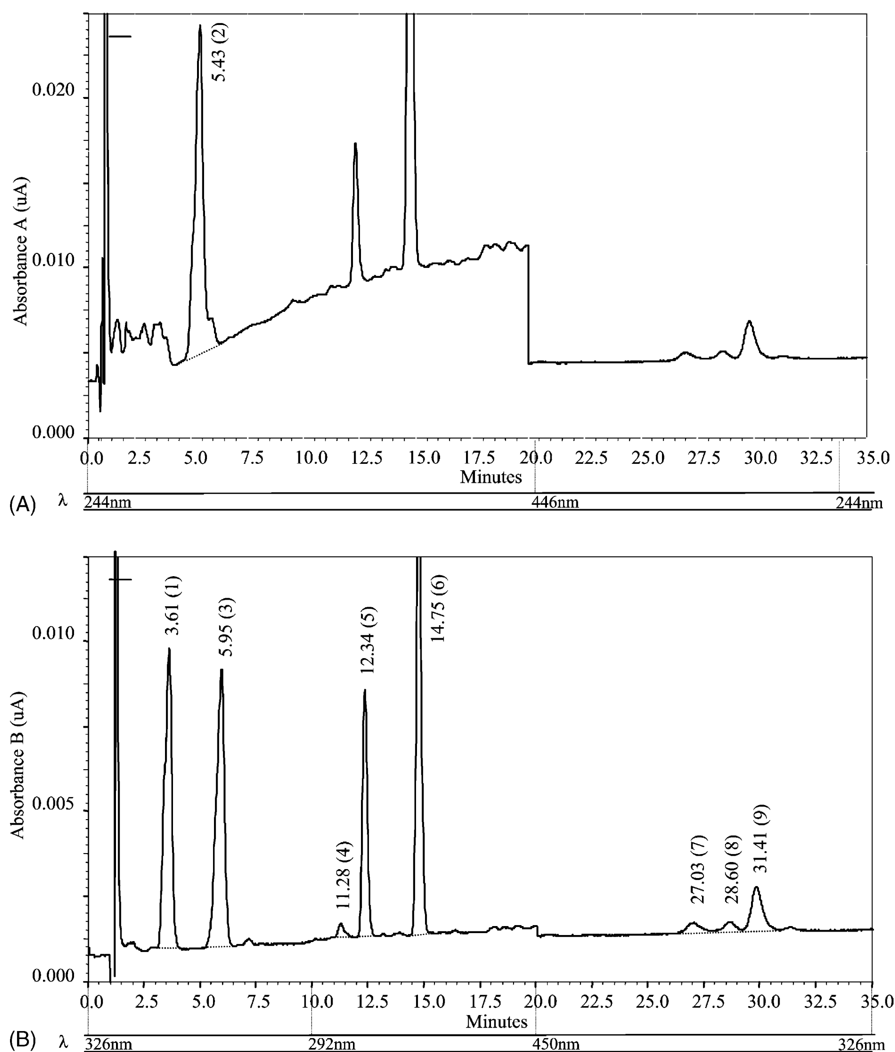

Fig. 1. Representative HPLC chromatogram of a human plasma sample. Reversed-phase HPLC conditions are given in Section 2. (A) Channel A, diode-array detection at 244 nm: probucol (2); (B) channel B, diode-array detection at 326 nm: retinol (1), retinol acetate (internal standard) (3); detection at 292 nm: γ-tocoferol (4), α-tocopherol (5), α-tocopherol acetate (internal standard) (6); detection at 450 nm: lycopene (7), α-carotene (8), β-carotene (9).

variation, nine injections in duplicate over a period of 12 h were made; for the inter-assay variation, two injections in each assay were made with a total of 22 assays.

2.7. Method validation

The standard reference material SRM 968c (fat soluble vitamins in human serum), from NIST (Gaithersburg, MD) was used as control.

2.8. Statistical methods

The results are presented as mean (standard deviation). Relationships between plasma and LDL antioxidants were evaluated by lineal regression analysis. Spearman correlation coefficients were calculated to evaluate relationships between variables. Statistical analyses were performed with a Statgraphics Plus v5.0 program (Statistical Graphics Corp.).

The concentration values are given as μg/ml; to convert into μmol/l, multiply retinol by 3.491, γ-tocopherol by 2.400, α-tocopherol by 2.322, lycopene, α-carotene and β-carotene by 1.863, and probucol by 1.935.

3. Results and discussion

3.1. Characteristics of the analytical system

The present HPLC procedure allowed a clear and rapid separation in a single run of the major lipid-soluble vitamins, as well as probucol and the two internal standards. Fig. 1 shows a representative chromatogram of the HPLC analysis of a human plasma sample; similar separations were obtained for isolated lipoprotein fractions. The compounds of interest were resolved within 35 min. Peak identification was achieved by comparison with the retention time and the spectrum of authentic standards. EDTA, which was used as anticoagulant for plasma isolation, showed no interference with any of the analytes (data not shown); this is important because in large-scale studies, the use of plasma versus serum is usually preferred.

The α-tocopherol acetate and retinol acetate were used as internal standards to monitor the possible losses caused during the extraction procedure of the samples; it is assumed that the added retinol acetate is equally extractable than the endogenous retinol and carotenes, whereas the added tocopherol acetate probes for the endogenous tocopherols [14,15]. Concentrations of analytes were calculated using a linear regression (area versus concentration) of each external standard curve, after correction for the contribution of endogenous analyte in the plasma employed. We observed a linear response over the concentration range for the calibration curves for all analytes, both in ethanol and in plasma, with correlation coefficients >0.99 in all cases (Table 1).

Table 1
Equations of the calibration curves used for the calculation of concentrations of the different analytes in samples

	Analytical range (μg/ml)	$y = ax + b$	r
Retinol	0.05–2.25	$y = 6.216x + 0.1318$	0.9992
γ-Tocopherol	0.137–2.50	$y = 0.246x + 0.0083$	0.9935
α-Tocopherol	0.906–47.0	$y = 0.179x + 0.0088$	0.9998
Lycopene	0.02–0.38	$y = 3.507x - 0.0201$	0.9944
α-Carotene	0.01–0.13	$y = 5.484x + 0.0065$	0.9986
All *trans*-β-carotene	0.02–0.38	$y = 5.061x - 0.0008$	0.9947
Probucol	1.5–45.0	$y = 1.759x + 0.7545$	0.9998

The calibration curve were calculated using the date from four independent assays, each one made up with six different concentration of the analytes.

Resolution of the peaks was satisfactory. Lycopene, α-carotene and β-carotene were not baseline-separated but resolution (0.87) and the separation was sufficient to allow for the individual quantitation of these compounds.

3.2. Recovery and stability of the analytes

Reconstitution of the residue after evaporation of hexane usually represents a difficulty because a highly non-polar solvent is needed to dissolve carotenes; it was decided to dissolve the residue in 200 μl of a mixture of propanol:acetonitrile (50:50, v/v). To calculate the recovery, 50 μl of the working combined standard solution at several concentrations were mixed with 50 μl of internal standard and 100 μl of ethanol. A fixed volume of 50 μl was injected directly into the cromatograph. The areas obtained were compared with plasma samples to which the same amounts of the antioxidants had been added and extracted as previously described. Additionally, we tested others matrixes as 0.9% sodium chloride and 0.01 M phosphate buffer, pH 7.4. As shown in Table 2, recovery of the different compounds were satisfactory in any of the matrixes used, the values being comparable to those reported by others [15–17]. However, taken all the analytes together, the best matrix appeared to be plasma.

Table 2
Recovery of analytes and internal standards from pooled plasma, sodium chloride and phosphate buffer samples

	Concentration added (μg/ml)	Recovery[a] ($n = 4$)		
		NaCl	Phosphate	Plasma
Retinol	0.670	87.0	79.3	98.1
γ-Tocopherol	1.03	109.7	109.2	108.3
α-Tocopherol	17.7	105.8	103.9	101.5
Lycopene	0.170	98.9	99.4	101.9
α-Carotene	0.060	108.6	114.1	93.8
β-Carotene	0.120	129.6	147.1	100.1
Probucol	13.3	100.5	98.9	100.1
Retinol acetate	0.500	82.1	81.4	96.8
α-Tocopherol acetate	8.00	106.0	114.7	100.2

[a] Recovery of analytes is expressed as percentage.

Table 3
Coefficients of variation of two control plasmas with different antioxidant concentrations (expressed as μg/ml)

	Mean (S.D.)	Confidence intervals (95%)	Intra-assay R.S.D. ($n = 9$)	Inter-assay R.S.D. ($n = 22$)
Level I				
Retinol	0.077 (0.005)	0.075–0.079	2.28	6.11
γ-Tocopherol	1.05 (0.08)	1.02–1.09	2.08	7.26
α-Tocopherol	7.62 (0.66)	7.32–7.93	1.02	8.65
Lycopene	0.025 (0.004)	0.023–0.026	7.80	15.90
α-Carotene	0.010 (0.002)	0.009–0.011	8.87	18.20
β-Carotene	0.044 (0.006)	0.042–0.048	4.40	12.70
Level II				
Retinol	0.552 (0.024)	0.543–0.561	2.13	4.36
γ-Tocopherol	0.784 (0.098)	0.747–0.822	2.18	12.50
α-Tocopherol	10.8 (0.54)	10.6–11.0	1.98	5.00
Lycopene	0.354 (0.019)	0.346–0.361	4.47	5.48
α-Carotene	0.087 (0.008)	0.084–0.090	5.89	8.87
β-Carotene	0.227 (0.018)	0.220–0.234	6.44	7.81

The measurement of the concentrations of the different compounds tested at the end and the beginning of each series, revealed that the plasma extracts dissolved in propanol:acetonitrile were stable for a minimum of 15 h, if placed in the autosampler at 4 °C; thereafter the concentration of lycopene and carotenes declined (data not shown).

3.3. Precision and sensitivity of the method

The calculated R.S.D.s of the procedure (Table 3) were comparable to those found in the literature [7,16]. The within-day precision (intra-assay R.S.D.) values were similar in the two plasma pools used, with different antioxidant concentrations, and was the best for α-tocopherol. As regards to the inter-assay precision, in general the higher the concentration of the analyte, the lower the day-to-day variation (Table 3).

The sensitivity of the method, based on a detection limit defined with a signal-to-noise ratio of 10:1, were as follows: 0.050 μg/ml for retinol, 0.137 and 0.906 μg/ml for γ- and α-tocopherol, respectively, 0.022 for lycopene, 0.008 and 0.150 μg/ml for α- and β-carotene, respectively, and 1.503 μg/ml for probucol. This sensitivity is sufficient for human plasma samples and even LDL fractions, since normal concentrations of the analytes of interest are well above these detection limits. If required, sensitivity could be augmented by increasing the sensitivity of the UV detector or by increasing the volume injected.

3.4. Method validation

Table 4 shows data obtained from the analysis of NIST standard reference material 968c. Results showed good agreement with certified values for retinol, α- and γ-tocopherol, lycopene, α- and β-carotene, in both reference samples.

3.5. Plasma and LDL levels of retinol, tocopherols, carotenoids and probucol

The procedure was used for measuring the concentrations of lipid-soluble antioxidants in plasmas and LDL fractions from patients treated with probucol. As shown in Table 5, the concentrations of retinol, γ-, α-tocopherol, lycopene, α-, β-carotene and probucol were within the expected range [16,17]. LDL accounted for most of the carotenoids—lycopene, α- and β-carotene—present in plasma, whereas only one-third of plasma tocopherol was confined to LDL, which is in agreement with previous findings in healthy populations reported by other [14,18,19]. Consistently with these results, positive and significant correlations between plasma and LDL concentrations were found for lycopene, α- and β-carotene but not for

Table 4
Results of analysis of NIST standard reference material 968c

	Certified NIST reference values	Observed values ($n = 5$)	
	Mean (S.D.)	Mean (S.D.)	Confidence intervals (95%)
Level I			
Retinol	0.841 (0.027)	0.819 (0.022)	0.792–0.846
γ-Tocopherol	3.90 (0.13)	3.25 (0.07)	3.16–3.34
α-Tocopherol	7.47 (0.47)	7.45 (0.16)	7.24–7.65
Lycopene	0.340 (0.040)	0.337 (0.011)	0.324–0.351
α-Carotene	0.020 (0.006)	0.021 (0.003)	0.017–0.025
β-Carotene	0.171 (0.017)	0.169 (0.006)	0.162–0.176
Level II			
Retinol	0.480 (0.012)	0.478 (0.012)	0.464–0.493
γ-Tocopherol	1.56 (0.10)	1.56 (0.11)	1.42–1.70
α-Tocopherol	16.8 (0.76)	16.2 (0.39)	15.7–16.7
Lycopene	0.450 (0.070)	0.454 (0.009)	0.442–0.466
α-Carotene	0.100 (0.020)	0.090 (0.004)	0.085–0.094
β-Carotene	0.436 (0.034)	0.397 (0.011)	0.383–0.411

Data are given in μg/ml.

Table 5
Mean concentrations and 95% confidence intervals for each of the variables measured in plasma and isolated LDL in patients treated with probucol

	Plasma (μmol/l plasma) ($n = 21$)		LDL (μmol/l plasma) ($n = 21$)	
	Mean (S.D.)	Confidence intervals	Mean (S.D.)	Confidence intervals
Retinol	2.81 (0.94)	2.40–3.23		
γ-Tocopherol	1.87 (1.01)	1.41–2.32	0.68 (0.32)	0.53–0.83
α-Tocopherol	26.0 (7.9)	22.4–29.6	9.32 (3.87)	7.55–11.08
Lycopene	0.192 (0.164)	0.116–0.266	0.175 (0.168)	0.099–0.252
α-Carotene	0.041 (0.053)	0.017–0.065	0.030 (0.039)	0.012–0.048
β-Carotene	0.162 (0.126)	0.105–0.220	0.124 (0.102)	0.078–0.171
Probucol	30.1 (26.7)	17.9–42.2	8.31 (7.32)	4.97–11.64

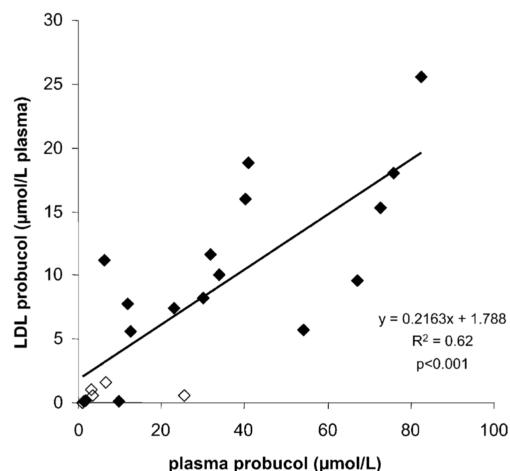

Fig. 2. Linear regression between probucol concentration in LDL and whole plasma in patients on treatment (solid symbols) or after drug withdrawal (open symbols).

α-tocopherol (data not shown). Other authors also reported the absence of correlation between tocopherol concentration in plasma and LDL [20,21].

LDL-probucol concentration was directly and significantly correlated with probucol concentration in whole plasma (Fig. 2). On average, LDL accounted for less than one-third of the total amount of probucol present in plasma (Table 5), meaning that the other lipoproteins contribute importantly to the transport of this drug.

As regards to the antioxidant content in LDL, positive and significant correlations were found between α- and γ-tocopherol ($r = 0.5304$, $P = 0.0134$, $n = 21$), between lycopene and α-tocopherol ($r = 0.4333$, $P = 0.0498$, $n = 21$), α-carotene ($r = 0.5517$, $P = 0.0095$, $n = 21$) and β-carotene ($r = 0.6944$, $P = 0.0005$, $n = 21$). Moreover, LDL-probucol content was directly correlated with that of α-tocopherol and lycopene (Fig. 3). The effect of probucol treatment on the plasma concentration of diet-derived antioxidants is controversial. Whereas in some studies probucol administration was accompanied by a decrease of the plasma levels of Vitamin E [22,23] and other lipophilic antioxidants [24], in other studies this could not be confirmed or even an increase in Vitamin E plasma concentration was observed [25,26]. Since lipid-soluble antioxidants are carried in plasma lipoproteins and, in general, direct correlations exist between their concentrations in plasma, the differences in the results mentioned above could be influenced by the changes in plasma lipids. Our results clearly indicate that on probucol treatment the LDL content of both tocopherol and lycopene increases. This may indicate that probucol protects the other antioxidants from destruction.

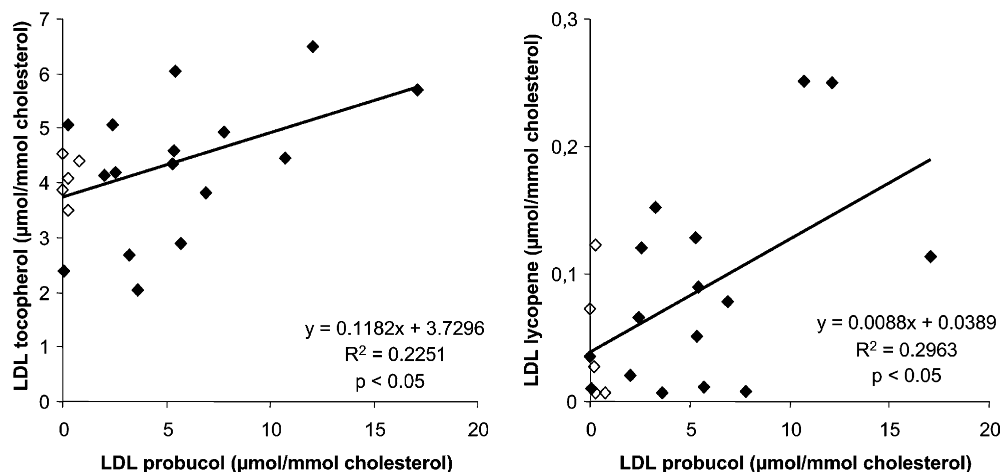

Fig. 3. Linear regression between the content of probucol and other antioxidants in LDL in patients on treatment (solid symbols) or after drug withdrawal (open symbols).

In any case, these results firmly recommend the measurement of the LDL content of lipophilic antioxidants in order to properly interpret the effects of vitamin supplements on the biological properties of these lipoproteins.

In conclusion, the method described in this paper allows the separation and quantitation of retinol, γ-tocopherol, α-tocopherol, lycopene, α-carotene, β-carotene and probucol in a single run, both in plasma and isolated lipoprotein fractions. The method gives excellent recoveries (≥97% for all analytes) and improved resolution for retinol, tocopherols and individual carotenoids. Photodiode array detection, with multiple wavelengths, is fully satisfactory for the identification and quantitation of these compounds. The analysis of lipid-soluble antioxidants in plasma showed good reproducibility and accuracy. This method opens up the possibility of including such biomarkers (tocopherols and carotenoids) in epidemiological studies with large number of subjects, where low costs, small sample volume and speed of analysis are key issues.

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