Simultaneous measurement of serum probucol and lipid-soluble antioxidants

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Abstract A method is described for the simultaneous measurement of probucol, retinol, tocopherols, lycopene, and carotenes by reverse phase high performance liquid chromatography. A high sensitivity was achieved by use of a microbore column and by monitoring the effluent at the optimum wavelengths of each substance with a diode array detector. The detection limits were lycopene 0.5 ng; α-carotene, β-carotene, and retinol 1 ng; probucol 2 ng; α-tocopherol and γ-tocopherol 15 ng. The eluent was acetonitrile–water–tetrahydrofuran 81.3:5.7:13 (v/v/v) and the flow rate was 0.4 ml/min. Quantitation was performed by use of the four internal standards retinol acetate, 2-pentanone bis(3,5-di-tert)mercaptole, α-tocopherol acetate, and retinol palmitate, which resemble the respective analytes in structure and/or polarity. In order to attain a reproducible recovery of particularly the carotenes, the total lipid content of the samples had to be controlled by dilution of the sample before extraction. The coefficients of variation for between-day determinations of a serum pool were 3.8% for retinol, 4.5% for probucol, 11.2% for γ-tocopherol, 4.5% for α-tocopherol, 10.4% for lycopene, 8.0% for α-carotene, and 7.0% for β-carotene.—**Schäfer Elinder, L., and G. Walldius.** Simultaneous measurement of serum probucol and lipid-soluble antioxidants. *J. Lipid Res.* 1992 **33:** 131–137.

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The biochemical mechanisms leading to the development of atherosclerosis are under intensive study. One hypothesis regarding the initiation of atherosclerosis is that of free radical-induced oxidation of lipoproteins, probably taking place in the arterial wall (1, 2). Oxidation of lipoproteins can be mediated by endothelial cells, smooth muscle cells, and monocytes, and may eventually lead to the formation of lipid-laden foam cells (1). This oxidative modification of lipoproteins can be inhibited by antioxidants in vitro. Esterbauer et al. (3) have shown that during in vitro oxidation of LDL a measurable oxidation of lipids did not take place before all natural antioxidants were consumed. The order of consumption was tocopherols, lycopene, and β-carotene. The Probucol Quantitative Regression Swedish Trial (PQRST), the design of which has been reported previously (4), was developed as a clinical trial to test whether probucol, a cholesterol-lowering drug that also has antioxidant properties (5), can retard the development or even cause regression of atherosclerosis. Probucol prevents copper-induced oxidation of human LDL in vitro in a concentration-dependent way (6). Thus, in vitro, the oxidation of lipoproteins can be inhibited by several lipid-soluble antioxidants. In vivo evidence in favor of this modified-LDL hypothesis has been presented by Carew (7), who showed that probucol inhibits the formation and progression of early atherosclerotic lesions in the spontaneously atherosclerotic Watanabe heritable hyperlipidemic rabbit.

In the PQRST study, we found it desirable to set up a method for measuring all the lipid-soluble antioxidants in the same serum sample in order to obtain a better estimate of the total antioxidant capacity of the patients at different stages of the trial. A recent review of the methodological aspects on the analysis of lipid soluble vitamins in serum by DeLeenheer et al. (8) served as a starting point.

We describe here a fast method for the quantitative extraction and analysis, by isocratic reverse phase high performance liquid chromatography (HPLC), of probucol, tocopherols, retinol, lycopene, and carotenes in human serum samples. Quantitation was performed by way of multiple internal standards as done by Kaplan, Miller, and Stein (9).

METHODS

Stock standards

The following standards were used: probucol and 2-pentanone bis(3,5-di-tert)mercaptole (PBM) were gifts from Marion Merrell Dow (Cincinnati, OH); DL-α-

Abbreviations: ACN, acetonitrile; HPLC, high performance liquid chromatography; PBM, 2-pentanone bis(3,5-di-tert)mercaptole; THF, tetrahydrofuran; LDL, low density lipoprotein; PAR, peak–area ratio.

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tocopherol and DL-α-tocopherol acetate were from Merck (Kebo, Stockholm, Sweden); α-carotene, retinol palmitate (all *trans*, synthetic), retinol acetate (all *trans*, synthetic), retinol (all *trans*, synthetic) were from Sigma (Lab Kemi, Stockholm, Sweden). β-Carotene (crystalline research grade) was from Serva (Lab Kemi, Stockholm, Sweden). It contained about 2% α-carotene. Lycopene (crystalline research grade) was a gift from Roche (Stockholm, Sweden). The standards were stored in amber ampules or flasks under nitrogen or argon at –20°C. The stock solutions were prepared in semidarkness, and the solvents were degassed with nitrogen before use. The following standards were prepared in 99.5% spectral grade ethanol (Kemetyl, Stockholm, Sweden): probucol (10 mg/ml), PBM (10 mg/ml), α-tocopherol (10 mg/ml), α-tocopherol acetate (10 mg/ml), retinol (2 mg/ml), retinol acetate (0.5 mg/ml). Retinol palmitate (10 mg/ml) was prepared in n-heptane (Merck, Kebo, Sweden). Lycopene (1 mg/ml) and β-carotene (1 mg/ml) were dissolved in chloroform (Merck, Kebo, Sweden). Probucol and PBM stock standards were prepared gravimetrically, whereas the exact concentrations of the other stock standards were determined by spectrophotometry. The instrument was a variable wavelength spectrophotometer (model DU-64, Beckman, Stockholm, Sweden). Relevant wavelengths, solvents, and absorptivities were adopted from Kaplan et al. (9). For α-tocopherol acetate the absorptivity ($E^{1\%}_{1cm}$ was 43.6 (10). The criterion of stability of the stock standard solutions was based on their spectra and absorbance and on the absence of degradation products as determined by HPLC. Probucol, PBM, α-tocopherol, α-tocopherol acetate, and retinol palmitate stock standards were prepared every 3 months and kept at 4°C in amber flasks. Retinol acetate, retinol, lycopene, and β-carotene stock standards were stable for at least 8 h. They were therefore prepared fresh, when making new working standard mixtures every 3–4 weeks.

Working standards

Working standard mixtures were prepared by diluting the stock standards in 99.5% ethanol. They were stored in amber flasks at 4°C. These working standards were used daily to check the stability of the HPLC system.

Combined internal standard I. This standard mixture was used for routine analysis of serum samples. The final approximate concentrations were: retinol acetate 0.25 μg/ml, PBM 10 μg/ml, α-tocopherol acetate 10 μg/ml, and retinol palmitate 0.5 μg/ml. This standard mixture was stable for 4 weeks, as determined by HPLC peak areas.

Combined internal standard II. This solution was used for the preparation of standard curves in a serum matrix. The approximate concentrations were: retinol acetate 0.5 μg/ml, PBM 20 μg/ml, α-TAC 20 μg/ml, and retinol palmitate 1 μg/ml.

Combined analyte standard. This solution was used for the preparation of standard curves in a serum matrix. The concentrations were: retinol 20 μg/ml, probucol 100 μg/ml, α-tocopherol 100 μg/ml, β-carotene 5 μg/ml, and lycopene 5 μg/ml. This solution was stable for 3 weeks, as determined by HPLC peak areas.

Chromatography

The HPLC system was from Hewlett Packard (Stockholm, Sweden), model HP 1090 series II, and consisted of a pump (PV5), a diode array detector, and an automatic sampler. The system was completely computerized (model HP 300, HP 9153C and HP 35741B). The analytical column was an ODS Hypersil, 5 μm, 200 × 2.1 mm (Hewlett Packard). The eluent composition was acetonitrile (ACN)–water–tetrahydrofuran (THF) 81.3:5.7:13 (v/v/v) and the flow rate was 0.4 ml/min. The solvents (HPLC grade) were continuously degassed with helium. In order to prevent changes in the eluent composition during degassing, ACN–H_2O and THF were delivered from two separate bottles. The column temperature was maintained at 40°C. Detection was performed with a diode array detector at six wavelengths simultaneously. The reference wavelength was set at 560 nm, with a band-width of 40 nm. The band-width for all detection wavelengths was set at 32 nm. The detection wavelengths were: probucol and PBM 244 nm; α-tocopherol acetate 285 nm; α-tocopherol and γ-tocopherol 292 nm; retinol, retinol acetate, and retinol palmitate 326 nm; lycopene, α-carotene and β-carotene 450 nm; and a metabolite of probucol 420 nm.

Standard curves, pure standards

Each stock standard was diluted 10, 20, 100, 250, and 500 times in ACN THF 50:50 (v/v). Ten μl of each dilution was injected. Peak areas were plotted as a function of the standard concentrations.

Patients

Fasting blood was obtained from patients in the prerandomization phase of the PQRST trial (4). At the time of blood sampling each patient had received regular diet counselling for 7 months, during which the intake of polyunsaturated fatty acids relative to saturated fatty acids (P/S ratio) had increased on average from 0.2 to 1.0. Medication consisted of 8–16 g of cholestyramine daily for 4 months and 1 g of

probucol daily for 2 months. The blood was allowed to coagulate for 2–3 h and the serum was frozen at –80°C within 3 h. The age of the samples at the time of analysis was 2–3 years. The average concentrations of cholesterol and triglycerides of these patients at the time of blood sampling were 6.31 ± 1.38 mmol/l and 1.87 ± 0.89 mmol/l, respectively. A fresh serum pool was made from a mixture of patient and normal serum and frozen at –80°C.

Extraction of serum samples

All samples were analyzed in duplicate. Individual serum samples were diluted with saline between 0.5–5 times (mostly between 1–3 times) to reach a total lipid content (cholesterol + triglycerides) of 4 to 6 μmol/ml. Thus the lipid content had to be known before the analysis for reasons given below. To 1.0 ml of diluted serum was added, dropwise while vortexing, 1.0 ml of the combined internal standard I in ethanol. Then 1.5 ml of n-heptane was added and the tube was capped. The tube was vortexed for 1 min at maximum speed and then centrifuged at 3000 rpm (Labofuge GL, Heraeus, Stockholm, Sweden) for 15 min. From the upper heptane phase, 1.3 ml was transferred to sample vials (32 × 11 mm). The solvent was evaporated under a stream of nitrogen at 40°C. The residue was reconstituted in 40 μl of ACN–THF 50:50 (v/v), vortexed, and transferred to microvials. These were firmly capped and enclosed in black sleeves, before being placed in the autosampler. Five μl of the extract was injected. Five serum samples could be extracted in duplicate in approximately 50 min by this procedure.

Standard curves in a human serum matrix

Standard curves were prepared every 3 – 4 weeks with the serum pool by the method of standard additions. All additions were performed in duplicate. The analyte/internal standard pairs were: retinol/retinol acetate; probucol/PBM; α-tocopherol/α-tocopherol acetate; lycopene/retinol palmitate; and β-carotene/retinol palmitate. Three dilutions of the serum pool were prepared in 0.9% saline to obtain total lipid concentrations (cholesterol + triglyceride) of 4, 5, and 6 μmol/ml. To 1.0 ml of diluted serum was added 1.0 ml of a mixture of the internal standards (0.5 ml of the combined internal standard II) plus either 0, 25, 50, 100, or 150 μl of the combined analyte standard plus ethanol to make a total of 1.0 ml. The samples were further processed as described above for serum samples. Standard curves were constructed with the peak area ratio (analyte/internal standard) (PAR) on the y-axis, and the amount of added analyte on the x-axis. When the correlation coefficient r was < 0.99 a new standard curve was made. The average slope value of the three curves was used for the calculation of unknown samples as described in the legend of **Fig. 1**.

RESULTS AND DISCUSSION

Chromatography

The response curves prepared by injecting dilutions of pure standards were linear with $r > 0.998$ for all substances (data not shown). Representative chromatograms of a serum sample with added internal standards are shown in **Fig. 2 a–e**. The chromatogram at 285 nm was very similar to that at 292 nm, and is therefore not shown. All substances were resolved within 16 min. The identification of substances was based on their retention time and spectrum. Retinol palmitate and β-carotene overlapped slightly in time, but this was no problem because they were detected at different wavelengths. α- And β-carotene were not baseline-separated. The resolution, calculated as $RS = 2*(V_{b\text{-}car}–V_{a\text{-}car})/(W_{b\text{-}car} + W_{a\text{-}car})$, where V is the retention time and W is the peak width, was 0.9. Others have reported a resolution of ca. 1–1.2 (11–13). However, as judged from the chromatogram at 450 nm, we regard the separation as sufficient to allow for the separate quantitation of the two substances.

In this type of analysis, where some components occur in the μg/l range in the original sample (carotenes), we found it advantageous to use a microbore

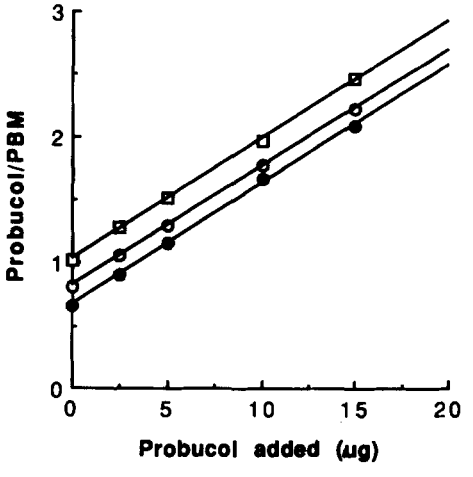

Fig. 1. Standard curves for probucol prepared by addition of known amounts to a human serum pool diluted to total lipid values of 4 μmol/ml (●), 5 μmol/ml (○), and 6 μmol/ml (□). Peak-area ratios (PAR) were calculated between the analyte probucol and its internal standard 2-pentanone bis(3,5-di-tert)mercaptole (PBM) and were plotted as a function of the amount of probucol added. The analyte concentration of an unknown sample was calculated as follows: [analyte] = (PAR_{sample}/a) × dilution factor, where a is the average slope value of the three curves. The slope value for γ-tocopherol was derived from that of α-tocopherol by multiplication with the ratio between their respective absorptivities. In the same way the slope for α-carotene was derived from that of β-carotene.

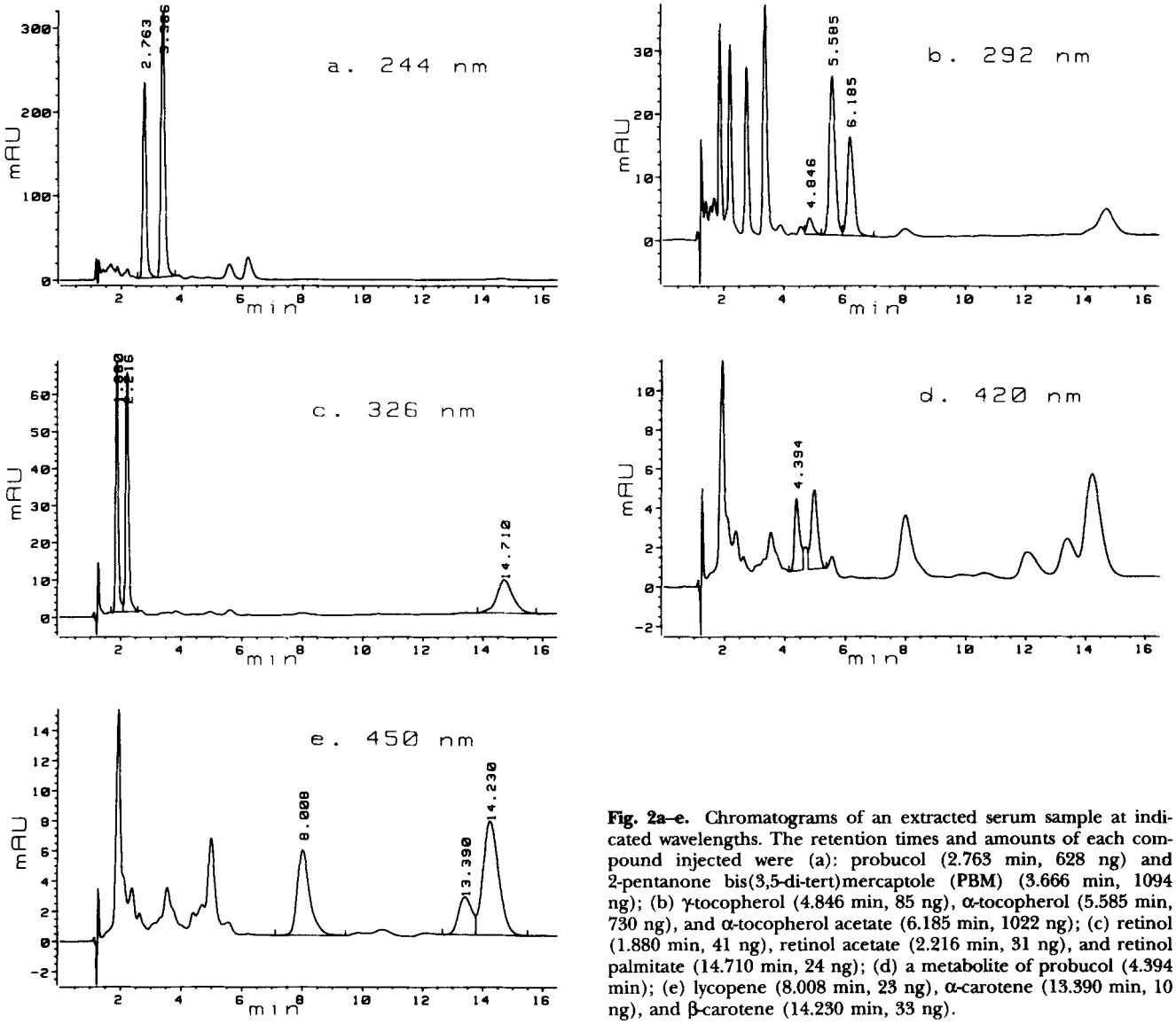

Fig. 2a–e. Chromatograms of an extracted serum sample at indicated wavelengths. The retention times and amounts of each compound injected were (a): probucol (2.763 min, 628 ng) and 2-pentanone bis(3,5-di-tert)mercaptole (PBM) (3.666 min, 1094 ng); (b) γ-tocopherol (4.846 min, 85 ng), α-tocopherol (5.585 min, 730 ng), and α-tocopherol acetate (6.185 min, 1022 ng); (c) retinol (1.880 min, 41 ng), retinol acetate (2.216 min, 31 ng), and retinol palmitate (14.710 min, 24 ng); (d) a metabolite of probucol (4.394 min); (e) lycopene (8.008 min, 23 ng), α-carotene (13.390 min, 10 ng), and β-carotene (14.230 min, 33 ng).

column with an internal diameter of 2.1 mm. Not only does this improve the separation of the different analytes, but it also greatly reduces solvent consumption. With a flow rate of 0.4 ml/min, solvent use is reduced 3- to 4-times compared to ordinary columns with an internal diameter of 4.6 mm. Because of the large number of samples to be analyzed, we decided to run the analysis isocratically, whereby long equilibration times between runs are avoided. However, this resulted in somewhat wider peaks for the late eluting carotenes. Other investigators have used a mobile phase containing chlorinated solvents for the separation of carotenes (9, 11, 12, 14–16). Because of the toxicity of such solvents, we tried various mixtures of ACN, water, and THF. The effect of THF was to achieve a high carotene dissolving power and to prevent precipitation of nonpolar compounds on the column. In order to achieve separation of the tocopherols and of retinol from retinol acetate, the water content had to be relatively high. A satisfactory separation was obtained with a mixture containing 13% THF and 5.7% water in ACN. The reconstitution of the residue after evaporation of the heptane usually presents a problem, because a highly nonpolar solvent needed to dissolve the carotenes (e.g., heptane) is not compatible with the mobile phase of the HPLC. An acceptable compromise was to dissolve the residue in 40 μl of ACN–THF 50:50 (v/v). A re-solution test with 40 μl ACN–THF 50:50 (v/v) showed that 2–5% of retinol, probucol, tocopherols, and their respective internal standards and about 10% of lycopene, carotenes, and retinol palmitate were left in the sample vial after the transfer of the extract to micro vials.

Detection

The diode array detector is excellent for this type of analysis, because all relevant wavelengths can be monitored simultaneously. Furthermore, even peaks that overlap in time can be quantitated, provided that their spectra do not overlap. With the use of this detector it is therefore possible to analyze several analyte/internal standard pairs at their optimum wavelengths and with optimal sensitivity. The spectrum facility on this detector allowed us to discover a metabolite of probucol in the serum extract, which is shown in Fig. 2d (420 nm) at 4.39 min. This peak was exclusively found in samples containing probucol. Based on the report by Barnhart, Busch, and Jackson (6) we tentatively identified it as an oxidation product of probucol, namely the diphenoquinone, which has its absorption maximum at 420 nm. The peak area of this compound constituted 1–2% of the probucol peak area. The concentration of this substance doubled within 6 h in the extracted sample dissolved in ACN–THF, but not in serum left to stand. No measurable decline in the concentration of probucol occurred during this period. This metabolite of probucol is probably generated both in vivo and in vitro.

The detection limits were calculated at a signal to noise ratio of 10:1 and were as follows: lycopene 0.5 ng; retinol, α-carotene, and β-carotene 1 ng; probucol 2 ng; and α-tocopherol and γ-tocopherol 15 ng. This sensitivity is comparable or better than that reported previously with ordinary UV detectors (12, 17).

Standardization

Due to differences in the recovery during sample extraction of compounds with differing polarity, the use of multiple internal standards with similar structure and/or polarity as the analytes is an improvement over older methods. Previously only a single internal standard such as α-tocopherol acetate or tocol has been used in this type of analysis (13, 14, 16, 18). The choice of internal standards was partly adopted from Kaplan et al. (9). Analyte concentrations of unknown samples were calculated by dividing the analyte/internal standard ratio (PAR) of the unknown sample by the slope value of the relevant standard curve prepared in a human serum matrix, as described in Fig. 1. Such a procedure requires that standard curves made from different serum pools are parallel. This was the case for retinol, probucol, and the tocopherols. However, for the carotenes, the recovery declined with an increasing lipid content of the serum samples. In other reports on the analysis of carotenes in human serum, this problem has not been addressed. We decided to keep the lipid content of the samples between 4 and 6 μmol/ml. Within this interval the slope of the standard curve for β-carotene varied up to 15%.

The recoveries of analytes and internal standards were determined from the standard curves, where known amounts were added in ethanol to the serum pool. The combined internal standard II, and the combined analyte standard were analyzed directly on the HPLC. Absolute recoveries of analytes and internal standards could hereafter be calculated and are given in **Table 1**. These values are comparable to those of Kaplan et al. (9). The recovery of probucol from serum was nearly twice as high as previously reported (19, 20). Representative average analyte concentrations from 40 patients are given in **Table 2**, as well as values on the precision of the method. The sensitivity and the precision of our method compares favorably with published results (9, 14, 16, 21, 22). The average concentration of the tocopherols, retinol, lycopene, and the carotenes of these patients are close to the 95th of that of normal subjects, reported by Kaplan et al. (23). The probucol concentration after 2 months of intake reported in previous studies has been 17.3 mg/l (20) and after 1 year of intake 19.0 mg/l (5). Our higher values are probably due to the higher recovery of probucol from serum by our extraction procedure. Therefore, our method gives a better estimate of the true serum probucol level.

Stability of analytes

The stability of the analytes in fresh serum was studied at time 0, 2 h, 4 h, 20 h, and 24 h. No decreases were noted in serum left to stand for 24 h in either darkness at 4°C, darkness at 20°C, or light at 20°C. The extracted samples, which were dissolved in 40 μl ACN–THF 50:50 (v/v) and placed in black sleeves in the autosampler, were stable for about 8 h. Thereafter the concentration of the carotenes started

TABLE 1. Recovery of analytes and internal standards from spiked serum samples

Substance	Amount Added	Recovery[a]
	μg	%
Retinol	2	86
Retinol acetate (ISTD)[b]	0.25	100
Probucol	10	96
PBM (ISTD)	10	93
α-Tocopherol	10	100
α-Tocopherol acetate (ISTD)	10	95
Lycopene	0.5	84
β-Carotene	0.5	77
Retinyl palmitate (ISTD)	0.5	100

[a]Values are averages of 3 experiments with a serum pool diluted with saline to attain a total lipid content of 4 μmol/ml.

[b]Internal standard.

TABLE 2. Representative average serum concentrations of analytes and methodological coefficients of variation

Substance	Serum Concentration[a]	Coefficient of Variation (%) Between-Day[b]	Coefficient of Variation (%) Between Duplicate Samples
Retinol	816 ± 157 μg/l (802)	3.8	2.3
Probucol	31.3 ± 16.7 mg/l (29.8)	4.5	1.6
γ-Tocopherol	1.88 ± 0.76 mg/l (1.74)	11.2	4.7
α-Tocopherol	17.0 ± 3.8 mg/l (16.6)	4.5	2.2
Lycopene	227 ± 117 μg/l (199)	10.4	2.3
α-Carotene	51.3 ± 44.1 μg/l (40.0)	8.0	4.1
β-Carotene	280 ± 242 μg/l (220)	7.0	2.4

[a]Values are mean ± SD of 40 serum samples analyzed in duplicate. Values in parentheses are geometric means.

[b]A serum pool was analyzed 30 times over a period of 4 months.

to decline. We found that it was not necessary to protect the sample from light during the extraction procedure. We obtained the same results for all analytes when extracting the sample in subdued or in full daylight. Other investigators carried out their analyses in dimmed or red light. As far as we can see, this is an unnecessary precaution. With regard to the long term stability of serum samples stored at −70°C, other investigators have concluded that tocopherols are stable in serum samples stored for up to 4 years (24), and that retinol and carotenes are stable for at least 28 months (10). Taken together, these results demonstrate the remarkable stability of these otherwise labile substances, when protected in a serum matrix.

Conclusion

In this report we have presented a rapid, sensitive, and precise chromatographic method for the simultaneous measurement of six lipid-soluble antioxidants and retinol in human serum. It compares favorably with established methods for the analysis of either one of the analytes in question. However, rigorous standardization is necessary. Fresh standard solutions of the most labile compounds should be prepared when new standard curves are constructed every 3–4 weeks, the maximum lifetime of the internal standard mixture. Approximately 20 serum samples, analyzed in duplicate, can conveniently be assayed in 24 h. This method is therefore suited for small as well as large clinical and epidemiological studies, such as the PQRST study (4), where lipid oxidation is of interest. The results of the PQRST trial will be reported elsewhere.

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REFERENCES

1. Steinberg, D., S. Parthasarathy, T. E. Carew, J. C. Khoo, and J. L. Witztum. 1989. Modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* **320:** 915–924.
2. Halliwell, B. 1989. Free radicals, reacitive oxygen species and human disease: a critical evaluation with special reference to atherosclerosis. *Br. J. Exp. Pathol.* **70:** 737–757.
3. Esterbauer, H., G. Striegl, H. Puhl, S. Oberreither, M. Rotheneder, M. El-Saadani, and G. Jürgens. 1989. The role of vitamin E and carotenoids in preventing oxidation of low density lipoproteins. In Vitamin E: Biochemistry and Health Implications. *Ann. N.Y. Acad. Sci.* **570:** 254–267.
4. Walldius, G., L. A. Carlson, U. Erikson, A. G. Olsson, J. Johansson, J. Mölgaard, S. Nilsson, G. Stenport, L. Kaijser, C. Lassvik, and I. Holme. 1988. Development of femoral atherosclerosis in hypercholesterolemic patients during treatment with cholestyramine and probucol/placebo: Probucol Quantitative Regression Swedish Trial (PQRST): a status report. *Am. J. Cardiol.* **62:** 37B–43B.
5. Buckley, M. M-T., K. L. Goa, A. H. Price, and R. N. Brogden. 1989. Probucol. A reappraisal of its pharmacological properties and therapeutic use in hypercholesterolaemia. *Drugs.* **37:** 761–800.
6. Barnhart, R. L., S. J. Busch, and R. L. Jackson. 1989. Concentration–dependent antioxidant activity of probucol in low density lipoproteins in vitro: probucol degradation precedes lipoprotein oxidation. *J. Lipid Res.* **30:** 1703–1710.
7. Carew, T. E. 1989. Role of biologically modified low–density lipoprotein in atherosclerosis. *Am J. Cardiol.* **64:** 18G–22G.
8. de Leenheer, A. P., H. J. Nelis, W. E. Lambert, and R.M. Bauwens. 1988. Chromatography of fat–soluble vitamins in clinical chemistry. *J. Chromatogr.* **429:** 3–58.

9. Kaplan, L. A., J. A. Miller, and E. A. Stein. J. 1987. Simultaneous measurement of serum retinol, tocopherols, carotenes, and carotenoids by high performance liquid chromatography. *Clin. Lab. Anal.* **1:** 147–152.
10. Craft, N. E., E. D. Brown, and J. C. Smith. 1988. Effects of storage and handling conditions on concentrations of individual carotenoids, retinol, and tocopherol in plasma. *Clin. Chem.* **34:** 44–48.
11. Broich, C. R., L. E. Gerber, and J. W. Erdman. 1983. Determination of lycopene, α- and β-carotene and retinyl esters in human serum by reversed-phase high performance liquid chromatography. *Lipids.* **18:** 253–258.
12. Olmedilla, B., F. Granado, E. Rojas-Hidalgo, and I. Blanco. 1990. A rapid separation of ten carotenoids, three retinoids, alpha-tocopherol and d-alpha-tocopherol acetate by high performance liquid chromatography and its application to serum and vegetable samples. *J. Liq. Chromatogr.* **13:** 1455–1483.
13. Tangney, C. C. 1984. Individual carotenoid determinations in human plasma by high-performance liquid chromatography. *J. Liq. Chromatogr.* **7:** 2611–2630.
14. Miller K. W., and C. S. Yang. 1985. An isocratic high-performance liquid chromatography method for the simultaneous analysis of plasma retinol, α-tocopherol and various carotenoids. *Anal. Biochem.* **145:** 21–26.
15. Gatautis V. J., and K. H. Pearson. 1987. Separation of plasma carotenoids and quantitation of β-carotene using HPLC. *Clin. Chim. Acta.* **166:** 195–206.
16. Thurnham, D. I., E. Smith, and P. S. Flora. 1988. Concurrent liquid–chromatographic assay of retinol, α-tocopherol, β-carotene, α-carotene, lycopene, and B-cryptoxanthin in plasma, with tocopherol acetate as internal standard. *Clin. Chem.* **34:**377–381.
17. Nierenberg, D. W. 1985. Serum and plasma β-carotene levels measured with an improved method of high-performance liquid chromatography. *J. Chromatogr. Biomed. Appl.* **339:**273–284.
18. Cavina, G., B. Gallinella, R. Porra, P. Pecora, and C. Suraci. 1988. Carotenoids, retinoids and alpha-tocopherol in human serum: identification and determination by reversed-phase HPLC. *J. Pharmacol. Biomed. Anal.* **6:** 259–269.
19. Satonin D. K., and J. E. Coutant. 1986. Comparison of gas chromatography and high-performance liquid chromatography for the analysis of probucol in plasma. *J. Chromatogr. Biomed. Appl.* **380:** 401–406.
20. Fellin, R., A. Gasparotto, G. Valerio, M. R. Baiocchi, R. Padrini, S. Lamon, E. Vitale, G. Baggio, and G. Crepaldi. 1986. Effect of probucol treatment on lipoprotein cholesterol and drug levels in blood and lipoproteins in familial hypercholesterolemia. *Atherosclerosis.* **59:** 47–56.
21. Milne, D. B., and J. Botnen. 1986. Retinol, α-tocopherol, lycopene, and α- and β-carotene simultaneously determined in plasma by isocratic liquid chromatography. *Clin. Chem.* **32:** 874–876.
22. Ito, Y., J Ochiai, R. Sasaki, S. Suzuki, Y. Kusuhara, Y. Morimitsu, M. Otani, and K. Aoki. 1990. Serum concentrations of carotenoids, retinol and α-tocopherol in healthy persons determined by high-performance liquid chromatography. *Clin Chim. Acta.* **194:** 131–144.
23. Kaplan, L. A., E. A. Stein, W. C. Willett, M. J. Stampfer, and W. S. Stryker. 1987. Reference ranges of retinol, tocopherols, lycopene and alpha- and beta-carotene in plasma by simultaneous high-performance liquid chromatographic analysis. *Clin. Physiol. Biochem.* **5:** 297–304.
24. Gunter, E. W., W. J. Driskell, and P. R. Yeager. 1988. Stability of vitamin E in long-term stored serum. *Clin. Chim. Acta.* **175:** 329–336.