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Simultaneous determination of retinol, α-tocopherol and β-carotene in serum by isocratic high-performance liquid chromatography

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ABSTRACT

A simultaneous determination of retinol, α-tocopherol and β-carotene in serum by high-performance liquid chromatography is desdribed. Total analysis time is 13 min. A reversed-phase (Ultrasphere ODS, 5 μm) column is used with a mobile phase of acetonitrile–methanol–dichloromethane (70:10:20, v/v/v) and a flow-rate of 1.2 ml/min. Retinol is monitored at 325 nm, α-tocopherol at 292 nm and β-carotene at 450 nm. Serum is deproteinized with ethanol containing the internal standard (α-tocopherol acetate), then extracted with hexane. The evaporated organic layer is reconstituted with the mobile phase and injected. The choice of the eluent is discussed, as well as the choice of an internal standard and the need for an antioxidant during the extraction step. Sixteen different eluents are compared in terms of analysis time and selectivity. The linear concentration ranges (retinol 0.016–13.7 μ*M*, α-tocopherol 0.18–91.8 μ*M*, β-carotene 0.05–5.75 μ*M*), within-run coefficients of variation (retinol <7%; α-tocopherol <8%, β-carotene <7%), between-run coefficients of variation (retinol <13%, α-tocopherol <9%, β-carotene <8%) and recoveries (retinol >95%, α-tocopherol >91%, β-carotene >80%) are suitable for clinical investigations. Serum reference values were found to be 2.47 ± 0.61 μ*M* (retinol), 30.5 ± 6.8 μ*M* (α-tocopherol) and 0.91 ± 0.55 μ*M* (β-carotene). A significant difference ($p < 0.001$) between males and females was found for retinol.

INTRODUCTION

Clincal interest in the simultaneous determination of retinol, α-tocopherol and β-carotene has increased in recent years, in relation to their scavenger effect against damaging free radicals [1]. As a result, determination of these three compounds is of interest in many pathological processes where a deficient protection system has been described (*e.g.* cancer, aging, cardiovascular diseases, alcoholism, cystic fibrosis, inflammatory and infectious diseases, AIDS) [1]. Thus, a rapid, sensitive, specific, precise and accurate method is of interest.

Several reversed-phase high-performance liquid chromatographic (HPLC) procedures have been described for the simultaneous determination of these three compounds [2–10]. However, some of them are time-consuming [4] or use an important flow-rate [3–6,10]. Others use multiple solvent systems [3,5,6,10], which require an additional delay for column reequilibration [7]. Several methods

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use the same wavelength (280 or 292 nm) to determine both retinol and α-tocopherol [2,4,6,7,10]. As a result, the sensitivity and specificity of retinol determination are reduced [7]. Moreover, the eluent choice is discussed in only few of them.

Nevertheless, an assay that could properly separate these three compounds, which allows a wavelength change, and sensitive and specific measurement of both retinol and α-tocopherol in a reasonable analysis time and with a reduced flow-rate, would be highly desirable.

This paper describes a rapid isocratic HPLC procedure which allows good separation of the three compounds within 13 min. Sixteen mobiles phases are compared in terms of analysis time and selectivity. To the best of our knowledge, it is the first report that compares different eluents for the simultaneous determination of retinol, α-tocopherol and β-carotene in serum. The choice of the internal standard and use of an antioxidant during the extraction step are also discussed. Moreover, the analytical performance of this method is clearly established.

EXPERIMENTAL

Apparatus

The chromatograph was from Kontron Instruments (Rotkreuz, Switzerland) and consisted of a solvent-delivery pump (LC pump, Model T414), a 50-μl injection valve (Model 7161, Rheodyne, Cotati, CA, USA), a guard-column packed with 10 μm Spheri-10 RP_{18} (3 cm × 0.46 cm I.D. from Applied Biosystems, Foster City, CA, USA), an analytical stainless-steel column packed with Ultrasphere ODS (5 μm) (15 cm × 0.46 cm I.D., from Beckman, San Ramon, CA, USA) and a multiwavelength detection system (HPLC detector 430). The system was entirely controlled by a computer (Data System 450).

The detector was programmed as follows: from 0 to 3.5 min at 325 nm to determine retinol, from 3.5 to 8 min at 291 nm to determine α-tocopherol and α-tocopherol acetate, and from 8.0 to 14 min at 450 nm to determine β-carotene. The baseline was automatically adjusted to zero with each wavelength change. The detector sensitivities were 0.01 a.u.f.s. (retinol detection at 325 nm) and 0.005 a.u.f.s. (α-tocopherol and α-tocopherol acetate detection at 292 nm, and β-carotene detection at 450 nm).

Injections were made with a 50-μl syringe from Hamilton (Bonaduz, Switzerland).

Reagents

Hexane and cyclohexane were fluorimetric grade (Merck, Darmstadt, Germany). Acetonitrile, dichloromethane and methanol were HPLC grade (Prolabo, Paris, France). Chloroform and 100% ethanol were Normapur grade (Prolabo). All these solvents were used without further purification. The mobile phases were degassed by sonication for 10 min prior to use.

Solutions of butylated hydroxytoluene (BHT) (Sigma, St. Louis, MO, USA) were prepared in 100% ethanol (0.125%) and in hexane (0.025%).

All-*trans*-retinol (7 m*M*, Sigma) and α-tocopherol (5 m*M*, U.S. Biochemicals Cleveland, OH, USA) were dissolved in 100% ethanol, and β-carotene (2.8 m*M*, Sigma) was dissolved in chloroform. These stock standard solutions were stored at −18°C, protected from light, for *ca.* two months. They were discarded when spectrophotometric determination (see below) revealed a decrease in concentration. They were diluted in 100% ethanol prior to analysis (retinol 28 μ*M*, α-tocopherol 400 μ*M*, β-carotene 14 μ*M*). The actual concentrations were determined using the specific absorbance ($A_{1cm}^{1\%}$: retinol, 1780 at 325 nm; α-tocopherol, 75.8 at 292 nm; β-carotene, 2396 at 465 nmn).

A working standard solution was prepared by mixing one volume of the three previous solutions and one volume of 100% ethanol. The two other standards were prepared by dilution in ethanol (1:1 and 1:3). The intermediate and working standard solutions were made daily.

A solution of α-carotene (Sigma) was also prepared in chloroform (1.9 m*M*), and then diluted in ethanol (7.4 μ*M*). For qualitative assays, 100 μl of this solution were mixed with 100 μl of the three working standard solutions.

A stock solution of the internal standard, 15 m*M* α-tocopherol acetate (U.S. Biochemicals), was prepared in 100% ethanol and stored at −18°C for three months, protected from light. The working internal standard solution (300 μ*M*) was prepared by dilution in 100% ethanol. Working internal standard solutions of 12 μ*M* retinyl acetate (Sigma), 12 μ*M* retinyl palmitate (Sigma), and 250 μ*M* α-tocopherol acid succinate (Sigma) were prepared in 100% ethanol.

Sample preparation

Blood was collected from the antecubital vein with a vacutainer (Becton Dickinson, Meylan, France) system and vacutainer tubes without anticoagulant. After centrifugation at 1600 *g* for 10 min, aliquots of serum were transferred to aluminium foil-wrapped polystyrene tubes and stored at −18°C until analysis, protected from light. Samples were analysed within fifteen days.

All the sample preparation was carried out in glass tubes (75 mm × 12 mm I.D.), protected by a sheet of aluminium, in order to minimize light-induced degradation of vitamins. A 100-μl volume of 100% ethanol and 100 μl of 100% ethanol containing the internal standard were added to 200 μl of serum. The mixture was stirred on a vortex-mixer for 5 s. The vitamins were then extracted with 500 μl of hexane by agitating on a vortex-mixer for 2 min. The tubes were centrifuged at 700 *g* for 5 min, and 250 μl of the hexane layer were transferred to another glass tube and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 200 μl of mobile phase, by mixing for 2 min, and 50 μl were immediately injected into the chromatograph.

The elution was carried out at room temperature. The flow-rate was 1.0, 1.2 or 1.5 ml/min, depending on the eluent.

TABLE I

EFFECT OF DIFFERENT ELUENTS ON THE RETENTION TIMES AND SELECTIVITY

The column was packed with 5 μm Ultrasphere ODS (15 cm × 0.46 cm I.D.).

Eluent	Flow-rate (ml/min)	Retention time (min)			R_s α/β-carotene
		Retinol	α-Tocopherol	β-Carotene	
1 Methanol	1.2	2.53	6.60	45.15	
2 Hexane–methanol (15:85, v/v)	1.0	2.14	3.33	10.51	0.87
3 Hexane–methanol (20:80, v/v)	1.0	1.95	2.75	7.67	0.58
4 Hexane–acetonitrile–methanol (12.5:42.5:45, v/v/v)	1.0	2.31	4.96	14.18	1.18
5 Hexane–acetonitrile–methanol (15:20:65, v/v/v)	1.0	2.24	4.05	12.67	1.02
6 Hexane–acetonitrile–methanol (15:30:55, v/v/v)	1.0	2.16	4.06	12.72	1.00
7 Hexane–acetonitrile–methanol (15:40:45, v/v/v)	1.0	2.04	4.00	12.96	1.16
8 Hexane–acetonitrile–methanol (17.5:37.5:45, v/v/v)	1.0	1.73	3.16	14.06	1.23
9 Hexane–acetonitrile–methanol (17.5:42.5:40, v/v/v)	1.0	1.48	2.74	18.85	1.30
10 Hexane–acetonitrile–methanol (18.2:27.3:54.5, v/v/v)	1.0	1.53	2.69	19.25	1.21
11 Hexane–acetonitrile–methanol (20:20:60, v/v/v)	1.0	1.85	2.92	9.82	1.04
12 Cyclohexane–acetonitrile–methanol (15:20:65, v/v/v)	1.0	2.27	4.20	13.94	1.34
13 Cyclohexane–acetonitrile–methanol (15:30:55, v/v/v)	1.0	2.27	4.60	16.26	1.55
14 Chloroform–acetonitrile–methanol (6:47:47, v/v/v)	1.5	1.99	5.56	18.85	1.34
15 Dichloromethane–acetonitrile–methanol (20:50:30, v/v/v)	1.0	2.60	5.40	12.54	1.06
16 Dichloromethane–acetonitrile–methanol (20:50:30, v/v/v)	1.2	2.11	4.23	9.51	0.70
17 Dichloromethane–acetonitrile–methanol (20:70:10, v/v/v)	1.0	2.61	5.87	12.84	1.19
18 Dichloromethane–acetonitrile–methanol (20:70:10, v/v/v)	1.2	2.31	5.31	11.79	1.11

Comparison of the retention times with those of standards established the peak identities. The concentrations of the analytes were determined by the peak-height ratio method.

Standard curve

The standard preparation was carried out in the same glass tubes: 100 μl of standard working solution and 100 μl of the internal standard solution were added to 200 μl of deionized water. The vitamins were extracted and determined, using the procedure described above.

Analytical performance

The detection limit (DL) was determined according to Gatautis and Pearson [11]. A sample, with vitamin concentrations of three to five times the noise level (retinol 0.115 μM, α-tocopherol 1.55 μM, β-carotene 0.29 μM) was measured ten times. The detection limit was calculated according to the formula $DL = (2 \times S.D. \times c)/S$ where S is the mean relative area, S.D. is the corresponding standard deviation and c is the concentration of the solution.

The linearity was established with correlation coefficients, according to the instructions of EEC (additive to directive 75/318/EEC, August 1989). Calibration standard solutions (retinol, 1.08, 5.6, 11.0, 16.7, 22.4 and 27.5 μM; α-tocopherol, 11.6, 23.3, 45.0, 92.4 and 183.6 μM; β-carotene, 0.6, 2.4, 5.9, 9.4 and 11.5 μM) were determined three times. The linear regressions and correlation coefficients were then calculated.

The precision and accuracy were determined according to the VALTEC protocol [12]. Three serum samples with different concentrations, available in our laboratory, were measured twice a day, for ten days, in order to calculate the within-run, using pair of values [12], and the between-run precision. For retinol determination, three pooled human sera, containing low (cystic fibrosis patients) (S1), normal (blood donors) (S2) and high (chronic renal failure patients) (S3) levels were selected. For β-carotene determination, low (cystic fibrosis patients) (S1), normal (blood donors) (S2) and high (bovine serum) (S4) level sera were also selected. For α-tocopherol determination, S4 was chosen as the low level serum, S2 was the normal level serum and a known amount (40 μM) of α-tocopherol, dissolved in ethanol, were added to serum S2 to give the high level serum. These sera were divided into aliquots and stored frozen (at $-20°C$). They were never exposed to thawing and refreezing.

The accuracy was evaluated by standard addition recoveries. First of all, known amounts of retinol, β-carotene and α-tocopherol, dissolved in chloroform–hexane, were added to pooled blood donors sera (retinol 2.7 μM, α-tocopherol, 50 μM, β-carotene, 0.63 μM). Secondly, a 100-μl aliquot of one of the three working standard solutions and 100 μl of the internal standard solution were added to 200 μl of serum, before extraction with hexane.

Normal values were established with samples from thirty-nine blood donors (nineteen males and twenty females).

RESULTS AND DISCUSSION

Optimization of the eluent

The isocratic mode was selected to reduce the delay between runs and the eluent consumption, and to increase column life [7].

The different standards were injected separately in order to examine the peak shapes and retention times. A serum sample was then injected. Both the analysis time and the presence of interfering compounds from plasma were examined. The separation of α- and β-carotenes was determined by the formula $R_s = 2[t_{R2} - t_{R1})/(W_2 + W_1)]$ where t_R = retention time of the compound (in min) and W the bandwidth determined by the intersection of the tangents to the inflection points of the Gaussian peak with the baseline (in min).

The use of methanol has been proposed by Milne and Botnen [8]. However, this eluent was judged inappropriate for simultaneous determination of retinol, α-tocopherol and β-carotene under our analytical conditions. The retention time of β-carotene was found to be 45.15 min (Table I). This eluent was too polar for the rapid elution of β-carotene [13]. Moreover, this solvent leads to on-column precipitation of carotenoids [2,13].

Different non-polar solvents were added to the mobile phase in order to improve the solubility of β-carotene [2,13]. However, the retention times of all the compounds were reduced and, with a high percentage of non-polar solvent, retinol and α-tocopherol are not sufficiently separated to allow a change of wavelength.

With the eluent used by Cavina *et al.* [2] (hexane–methanol 15:85, v/v) and a

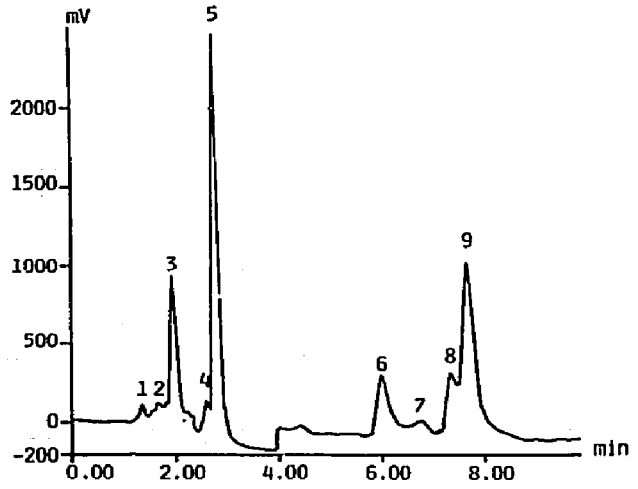

Fig. 1. Separation of retinol, α-tocopherol and β-carotene in serum on 5 μm Ultrasphere ODS (15 cm × 0.46 cm I.D.). Eluent, hexane–methanol (20:80, v/v); flow-rate, 1.0 ml/min. Peaks: 3 = retinol (detection at 325 nm); 5 = α-tocopherol (detection at 291 nm); 8 = α-carotene (detection at 450 nm); 9 = β-carotene (detection at 450 nm); 1, 2, 4, 6, 7 = unidentified peaks.

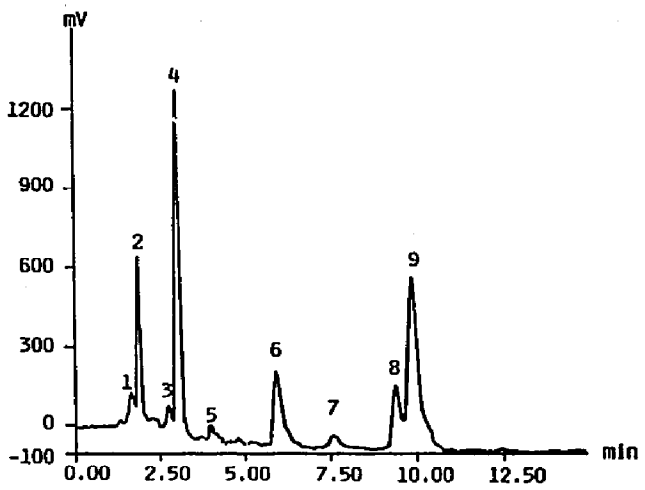

Fig. 2. Separation of retinol, α-tocopherol and β-carotene in serum on 5 μm Ultrasphere ODS (15 cm × 0.46 cm I.D.). Eluent, hexane–acetonitrile–methanol (20:20:60, v/v/v; flow-rate, 1.0 ml/min. Peaks: 2 = retinol (detection at 325 nm); 4 = α-tocopherol (detection at 291 nm); 8 = α-carotene (detection at 450 nm); 9 = β-carotene (detection at 450 nm); 1, 3, 5–7 unidentified peaks.

flow-rate of 1.0 ml/min, t_R for β-carotene was found to be 10.51 min (Table I), which is in agreement with the value of Cavina *et al.* [2]. This time was reduced to 7.67 min with a hexane–methanol (20:80, v/v) mobile phase and a flow-rate of 1.0 ml/min. Nevertheless, α-and β-carotene were not completely separated (R_s = 0.87 and 0.58, respectively, Table I) and unidentified compounds interfered with retinol (peak 2, Fig. 1) and α-tocopherol (peak 4, Fig. 1). A reduction of the flow-rate to 0.5 ml/min, after the elution of α-tocopherol, failed to resolve β- and

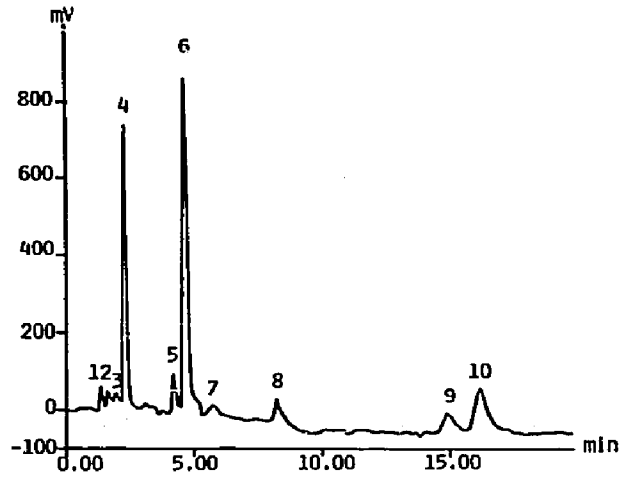

Fig. 3. Separation of retinol, α-tocopherol and β-carotene in serum on 5 μm Ultrasphere ODS (15 cm × 0.46 cm I.D.). Eluent, cyclohexane–acetonitrile–methanol (15:30:55, v/v/v); flow-rate, 1.0 ml/min. Peaks: 4 = retinol (detection at 325 nm); 6 = α-tocopherol (detection at 291 nm); 9 = α-carotene (detection at 450 nm); 10 = β-carotene (detection at 450 nm); 1–3, 5, 7, 8 = unidentified peaks.

α-carotenes, and the retention time of β-carotene was dramatically increased [t_R = 19 min, with hexane–methanol (15:85, v/v)].

According to Sowell *et al.* [14], acetonitrile improves the separation and peak height of carotenoids, but acetonitrile and hexane are immiscible, and acetonitrile increases the retention time of carotenoids [13]. Different ternary mixtures, composed of hexane, methanol and acetonitrile, were tried as the mobile phase (Table I). As reported by Nelis and De Leenheer [13], an increase of the acetonitrile/methanol ratio (Table I, eluents 5 to 7 and 8 to 9) increased the retention time of β-carotene. In contrast, the separation of β- and α-carotene was more efficient. These results were in agreement with previous studies [13,14]. An increase of the proportion of hexane (Table I, mobile phases 4 and 9, 5 and 11, 6 and 10, 7 and 8) decreased the retention times of retinol and α-tocopherol. On an other hand, with eluents 8–11, retinol was not separated from an unidentified interfering compound from serum (peak 1, Fig. 2). Finally, retinol was not sufficiently separated from α-tocopherol to allow a change of wavelength.

With cyclohexane, β- and α-carotenes were well separated (Table I). However, an unidentified interfering compound from serum slightly interfered with α-tocopherol (peak 5, Fig. 3).

Chloroform has been used by several authors to separate retinol, α-tocopherol and carotenoids [4,6,7,9,15,16]. A good separation was obtained with chloroform–acetonitrile–methanol (6:47:47, v/v/v), but the retention time of β-carotene was 18.85 min, with the column described above, even with a flow-rate of 1.5 ml/min (Table I, eluent 14). These results are different from those of Thurnham *et al.* [9], however, the analytical parameters are different.

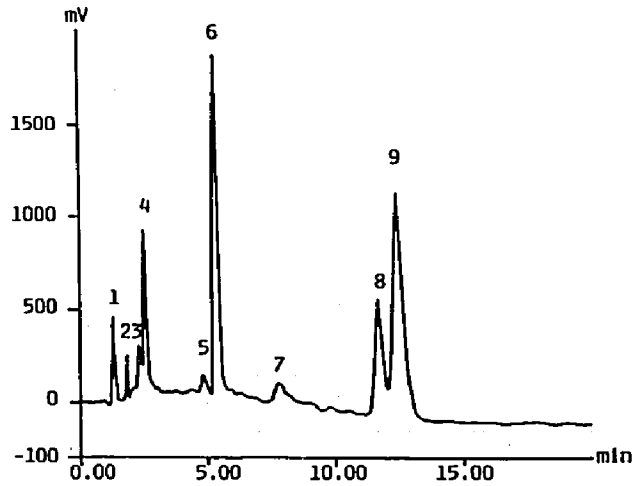

Fig. 4. Separation of retinol, α-tocopherol and β-carotene in serum on 5 μm Ultrasphere ODS (15 cm × 0.46 cm I.D.). Eluent, dichloromethane–acetonitrile–methanol (20:50:30, v/v/v; flow-rate, 1.0 ml/min. Peaks: 4 = retinol (detection at 325 nm); 6 = α-tocopherol (detection at 291 nm); 8 = α-carotene (detection at 450 nm); 9 = β-carotene (detection at 450 nm); 1–3, 5, 7 = unidentified peaks.

Dichloromethane has been used by several authors [11,13,17] to separate carotenoids. This solvent is more polar than chloroform, is an excellent solvent for carotenoids [13] and is miscible with acetonitrile. The dichloromethane–acetonitrile–methanol (20:50:30, v/v/v) eluent allowed a resolution of the three compounds within 13 min, with a flow-rate of 1.0 ml/min (Table I). However, an unidentified interfering compound from serum eluted just before the retinol peak (peak 3, Fig. 4). Substitution of some of the methanol by acetonitrile slightly increased the retention times but the resolution of α- and β-carotene was improved (Table I). These observations have been noted by Nelis and De Leenheer [13]. As a result, acetonitrile–dichloromethane–methanol (70:20:10, v/v/v) was retained for further assays. An increase of the flow-rate to 1.2 ml/min reduced the analysis time without decreasing the selectivity (Table I, Fig. 5). With this eluent and flow-rate the resolution was sufficient to allow determination of the three compounds in reasonable time (13 min).

Choice of an internal standard

Using a multiwavelength detector, a single internal standard is adequate, even through quantitation is made at different wavelengths [4,6].

We assayed different substances previously used (retinyl acetate [4–6,8,15,18], retinyl palmitate [4], α-tocopherol acetate [2,4,5,9,19] and α-tocopherol acid succinate]. Retinyl palmitate was unsuitable: it has a maximum absorption at 325 nm an does not absorb at 450 nm. The retention times of retinyl palmitate and β-carotene were 13.2 and 11.8 min, respectively, so the wavelength change was

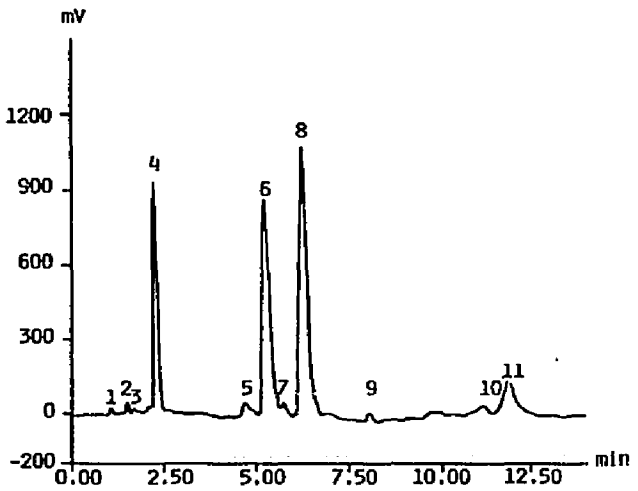

Fig. 5. Separation of retinol, α-tocopherol and β-carotene in serum on 5 μm Ultrasphere ODS (15 cm × 0.46 cm I.D.). Eluent, dichloromethane–acetonitrile–methanol (20:70:10, v/v/v; flow-rate, 1.2 ml/min. Peaks: 4 = retinol (detection at 325 nm); 6 = α-tocopherol (detection at 291 nm); 8 = α-tocopherol acetate (detection at 291 nm); 10 = α-carotene (detection at 450 nm); 11 = β-carotene (detection at 450 nm); 1–3, 5, 7, 9 = unidentified peaks.

TABLE II

RETENTION TIMES OF DIFFERENT SUBSTANCES USED AS INTERNAL STANDARDS

The column was packed with 5 μm Ultrasphere ODS (15 cm × 0.46 cm I.D.); eluent, dichloromethane–acetonitrile–methanol (20:70:10, v/v/v); flow-rate: 1.2 ml/min.

Substances	Retention time (min)
Retinyl acetate	2.60
Retinyl palmitate	13.2
α-Tocopherol acid succinate	4.96
α-Tocopherol acetate	6.30

not possible and the analysis time was increased (Table II, Fig. 6). This result is in agreement with previous studies [4,15]. α-Tocopherol acid succinate gave a distorted peak, probably related to its acid function (Fig. 6). Moreover, its retention time was very close to that of α-tocopherol. The resolution of both retinol and retinyl acetate was suitable, as reported previously [4,6,8,15,18]. α-Tocopherol acetate was also well separated from the other substances, as previously described [2,4,19]. The latter was chosen for practical reasons. Retinyl acetate is sensitive to light and oxygen, whereas α-tocopherol acetate is practically unaffected by the oxidizing influence of oxygen and light [18]. Finally, the retention time of α-tocopherol acetate (6.30 min) was more appropriate.

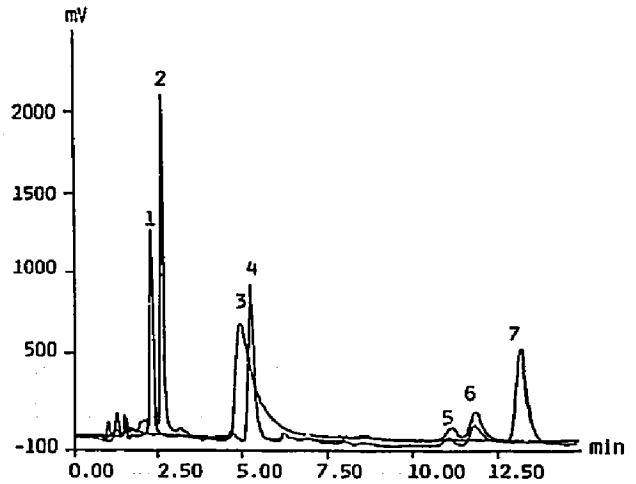

Fig. 6. Retinol, retinol acetate, α-tocopherol, α-tocopherol acid succinate, α-carotene, β-carotene and retinol palmitate profile on 5 μm Ultrasphere ODS (15 cm × 0.46 cm I.D.). Eluent, dichloromethane–acetonitrile–methanol (20:70:10, v/v/v); flow-rate, 1.2 ml/min. Peaks: 1 = retinol (detection at 325 nm); 2 = retinol acetate (detection at 325 nm); 3 = α-tocopherol acid succinate (detection at 291 nm); 4 = α-tocopherol (detection at 291 nm); 5 = α-carotene (detection at 450 nm); 6 = β-carotene (detection at 450 nm); 7 = retinol palmitate (detection at 325 nm).

TABLE III

INFLUENCE OF ANTIOXIDANT (BHT) ON THE CONCENTRATION OF RETINOL, α-TOCOPHEROL AND β-CAROTENE IN SERUM

The column was packed with 5 μm Ultrasphere ODS (15 cm × 0.46 cm I.D.); eluent, dichloromethane–acetonitrile–methanol (20:70:10, v/v/v); flow-rate, 1.2 ml/min. Results are expressed as mean ± S.D. (n = 5).

	Concentration (μM)		
	Retinol	α-Tocopherol	β-Carotene
With BHT	2.3 ± 0.5	27.5 ± 2.0	0.42 ± 0.38
Without BHT	2.4 ± 0.5	27.5 ± 3.5	0.50 ± 0.42

TABLE IV

WITHIN-RUN AND BETWEEN-RUN PRECISION

The column was packed with 5 μm Ultrasphere ODS (15 cm × 0.46 cm I.D.); eluent, dichloromethane–acetonitrile–methanol (20:70:10, v/v/v); flow-rate, 1.2 ml/min. Results are expressed as mean ± S.D. (n = 20). Values in parentheses are C.V. (%).

Serum	Concentration (μM)			
		Retinol	α-Tocopherol	β-Carotene
Low	WR[a]	0.87 ± 0.06 (7)	6.6 ± 0.5 (7.5)	0.34 ± 0.02 (5)
	BR	0.87 ± 0.11 (13)	6.6 ± 0.6 (9)	0.34 ± 0.03 (8)
Normal	WR	2.02 ± 0.05 (2.5)	26.6 ± 1.1 (4)	0.74 ± 0.05 (7)
	BR	2.02 ± 0.13 (6.5)	26.6 ± 1.7 (6)	0.74 ± 0.05 (7)
High	WR	5.60 ± 0.16 (3)	64.2 ± 2.2 (3.5)	3.01 ± 0.09 (3)
	BR	5.60 ± 0.35 (6)	64.2 ± 3.3 (5)	3.01 ± 0.22 (8)

[a] WR, within-run precision; BR, between-run precision.

TABLE V

RECOVERY OF STANDARD ADDITIONS

The column was packed with 5 μm Ultrasphere ODS (15 cm × 0.46 cm I.D.); eluent, dichloromethane–acetonitrile–methanol (20:70:10, v/v/v); flow-rate, 1.2 ml/min.

	Recovery (mean ± S.D.) (%)		
	Retinol	α-Tocopherol	β-Carotene
Addition of known amounts in ethanol (n = 30)	95 ± 11	91 ± 7	93 ± 8
Addition of known amounts in serum (n = 10)	105 ± 21	100 ± 18	80 ± 21

Effect of antioxidant

Some authors add an antioxidant to stabilize vitamins during the extraction process [5,9,19,20,21]. We used the procedure described by Chow and Omaye [20]. BHT was added to ethanol (0.125%) and hexane (0.025%). On the one hand, BHT was eluted near the retinol peak and thus interfered with its determination. This result is in agreement with previous reports [8,9]. On the other hand, we found no difference in the concentrations for retinol, α-tocopherol and β-carotene, as compared with the same sera extracted without added BHT (Table III), in agreement with previous results [3,8]. However, Singkamani *et al.* [22] have shown a positive effect of BHT on β-carotene sensitivity and Chow and Omaye [20] have noted a beneficial effect on recoveries. In conclusion, the use of an antioxidant during the extraction and drying steps seemed unnecessary.

Analytical performance

The detection limit was found to be 0.016 μM for retinol, 0.18 μM for α-tocopherol and 0.05 μM for β-carotene. These limits demonstrate the excellent sensitivity of the proposed method. The linear ranges were found to be 13.7 μM (r = 0.997) for retinol, 91.8 μM (r = 0.998) for α-tocopherol and 5.75 μM (r = 0.997) for β-carotene. These ranges allow the determination of the three compounds in specimens from almost all patients.

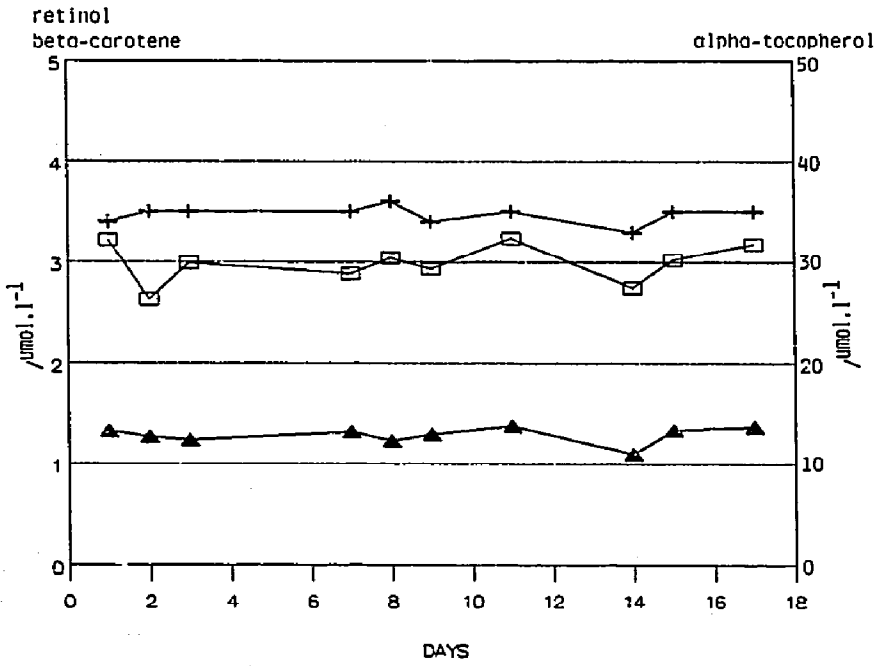

Fig. 7. Comparison of retinol, α-tocopherol and β-carotene concentrations in sera stored at −20°C for eighteen days. Column, 5 µm Ultrasphere ODS (15 cm × 0.46 cm I.D.); eluent, dichloromethane–acetonitrile–methanol (20:70:10, v/v/v); flow-rate, 1.2 ml/min. Results expressed as mean values in μM (n = 5): (□) retinol; (+) α-tocopherol; (▲) β-carotene.

TABLE VI

NORMAL VALUES OF RETINOL, α-TOCOPHEROL AND β-CAROTENE IN SERUM

The column was packed with 5 μm Ultrasphere ODS (15 cm × 0.46 cm I.D.); eluent, dichloromethane–acetonitrile–methanol (20:70:10, v/v/v); flow-rate, 1.2 ml/min.

n	Concentration (μ*M*)		
	Retinol	α-Tocopherol	β-Carotene
39	2.47 ± 0.61	30.5 ± 6.8	0.91 ± 0.55
19 males	2.81 ± 0.48	32.15 ± 7.6	0.79 ± 0.54
20 females	2.15 ± 0.55	29.0 ± 5.7	1.03 ± 0.56

The within-run and between-run coefficients of variation (C.V.) are indicated in Table IV. They ranged from 2.5 to 13%, which is within the degree of variation expected for this type of analysis, except for retinol at low level. Our C.V. are similar to those of previous studies at similar concentrations [2,4,7–9,11,15,18,19].

The standard recoveries were quite good, except for β-carotene added to serum (Table V). This low recovery for β-carotene can be easily explained: it has a tendency to cling to the side of the tube and is not easily mixed into the serum. Kaplan *et al.* [4] reported similar results, whereas others have reported excellent recoveries [3,6,11,15,16].

The between-run and recovery assays were conducted during an eighteen-day period. During this period, the stability was good (Fig. 7) for the three compounds, as shown by the correlation coefficients ($r < 0.03$). Several authors have shown that fat-soluble vitamins are stable in human serum for longer periods [3,5,6,10,21,23].

Normal values

The values for sera from thirty-nine blood donors are indicated in Table VI. No difference between male and female was found for β-carotene and α-tocopherol, but a significant difference ($p < 0.001$) was found for retinol, in agreement with previous studies [24].

CONCLUSION

The present method is specific, sensitive, precise and accurate. The small serum requirement (200 μl) allows determination in sera from infants and young children. The importance of this work is the comparison of sixteen different mobile phases. The mobile phase was carefully selected in order to achieve maximum separation in a minimum time. The isocratic elution allows a minimum of down-

time. Routinely, three samples can be analysed every hour, with 20 ml of mobile phase per assay. The same column can be used for *ca.* six months.

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