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Development and validation of a fast and sensitive chromatographic assay for all-*trans*-retinol and tocopherols in human serum and plasma using liquid–liquid extraction

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Abstract

A sensitive HPLC assay for all-*trans*-retinol, α-tocopherol, and γ-tocopherols in human serum and plasma is reported. Sample preparation is performed in one step and involves precipitation of proteins and extraction of lipids with two volumes of an ethanol–chloroform mixture (3:1, v/v) without I.S. addition. After removal of the precipitated protein, 20 μl aliquots of the supernatant (equivalent to 6.7 μl of serum or plasma) were injected into the HPLC system and analyzed using fluorometric detection. RP-HPLC was performed using a C_{18} S3 ODS2 column with a methanol–water step gradient (97:3 to 100) at 1.0 ml/min. The quantification limit expressed as nanograms of analyte per milliliter of serum or plasma was approximately 30 ng for all-*trans*-retinol, 300 ng for α-tocopherol and 250 ng for γ- and δ-tocopherol. The method was validated and applied to human serum and plasma from a total of 120 subjects. This procedure requires a small volume of serum or plasma and can therefore be a valuable tool for measuring low concentrations of these vitamins in preterm infants with sensitivity, precision and accuracy.

Keywords: Retinol; Tocopherols

1. Introduction

Vitamin A is crucial for morphogenesis, vision, immune function, reproduction, neuronal and neural development and maintenance of differentiation functions [1–3]. Recent interest has focused on the potential role of vitamin A status in modulating the effects of HIV infection, particularly in the vertical transmission from mother to infant [4]. In the last few years there has been growing clinical interest in the relationship between antioxidant micronutrients, such as vitamin E, in several degenerative human health conditions [5], and retinol deficiency with abnormal retinoid metabolism, which has been implicated in neuro degeneration associated with aging and with certain brain pathologies such as Alzheimer's diseases [6,7]. In addition, recent epidemiological and clinical studies have suggested that the fat-

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soluble vitamins A and E protect against coronary heart disease and they may be important in the prevention of some cancers [8–10]. Reduced plasma α-tocopherol levels have also been reported in the Smith–Lemli–Opitz syndrome [11].

Moreover, monitoring the status of vitamins A and E is important in detecting an inadequate dietary intake of these vitamins or a possible deficiency of either retinol-binding protein, protecting retinol from degradation and cells from its amphypathic properties, or of α-tocopherol transfer protein (α-TTP), which, in the liver, incorporates α-tocopherol into very-low-density lipoproteins [12]. Many methods have been reported for the simultaneous determination of vitamins A and E in serum or plasma [13–18] and various procedures for sample pretreatment have been developed [19], some based on protein precipitation using an organic solvent such as ethanol, isopropanol or acetonitrile, followed by liquid–liquid extraction. Extraction reagents include *n*-hexane [16], petroleum ether [20], dichloromethane–methanol [21], chloroform and acetone–chloroform [22], etc., with the addition of antioxidants such as butylated hydroxytoluene [16] or ascorbic acid [23] to avoid oxidation of fat-soluble vitamins. On the other hand, handling the organic phase by evaporation and reconstitution of the dried samples before HPLC injection results in low accuracy with a large variation [22] and is time-consuming. Furthermore, the use of an internal standard is a prerequisite to compensate for losses during the various handling steps.

In this paper we report a procedure for the simultaneous detection of vitamins A and E consisting of a one-step extraction without I.S. addition, direct injection of the sample into the RP-HPLC system and analysis.

2. Experimental

2.1. Chemicals

All-*trans*-retinol, DL-α-tocopherol, (+)-γ-tocopherol and (+)-δ-tocopherol were purchased from Sigma–Aldrich (Milan, Italy). The lyophilized control human serum (levels I and II) kit for HPLC analysis of vitamins A and E in serum and plasma and amber conical autosampler vials were purchased from Chromsystems Instruments and Chemicals (Munich, Germany). HPLC-grade chloroform, methanol ethanol and isopropanol were obtained from Carlo Erba (Milan Italy). HPLC-grade water was prepared from demineralized water using a Milli-Q Plus water purification system (Millipore, Milford, MA, USA).

2.2. Instruments

The analytical liquid chromatograph used throughout this work consisted of two Model 306 pumps, one 811B dynamic mixer, a Model 234 autoinjector equipped with a 20 μl injection loop, a 122 fluorometric detector, and Unipoint System Controller Software (v 1.71) for acquisition and elaboration of data (Gilson Italia, Milan, Italy). Separations of all-*trans*-retinol and tocopherols were performed using a Waters Spherisorb C_{18} S3 ODS2 column (15×0.46 cm I.D., 3 μm particle size) coupled to a guard column (3×0.46 cm I.D., RP C_{18}, 5 μm particle size) (Waters, Tauton, MA, USA).

2.3. Preparation of standards

Individual stock solutions of all-*trans*-retinol, α-tocopherol, δ-tocopherol and γ-tocopherol were prepared in absolute ethanol and stored at −80 °C, at which they were stable for approximately 2 months. Dilution was performed immediately prior to use. Absorbance was determined using a Beckman DU8 spectrophotometer and concentrations were calculated from the standard absorbance *E* (1 cm/1%): all-*trans*-retinol, 1780 at 325 nm; α-tocopherol, 75.8 at 292 nm; γ- and δ-tocopherol, 92.8 and 91.2, respectively, at 298 nm [24].

For calibration curves the stock solutions were diluted with water, ethanol and chloroform (final ratio 2:3:1, v/v/v) to obtain a mixture of working standards whose concentration was in the range expected in serum or plasma ±50%. Because, as reported in Section 2.5, a 1:2 dilution of serum or plasma was made to perform vitamin extraction, the concentrations of the working standards, expressed as ng×20 μl^{-1}, correspond to those present in 6.67 μl of the original serum or plasma sample. Thus, working standard concentrations of 1, 2, 3, 4 and 8

ng of all-*trans*-retinol, 10, 20, 30, 90 and 180 ng of α-tocopherol and 1, 3, 6, 8 and 10 ng of γ-tocopherol correspond to 0.52–4.2, 3.48–62.64 and 0.36–3.6 μmol/l, respectively.

2.4. Blood collection and storage

Blood samples from 10 healthy male volunteers (aged 28–65 years) on non-supplemented diets were obtained by venipuncture using vacuum tubes with or without EDTA. The study was performed in accordance with the Helsinki Declaration of 1975, as revised in 1989. The tubes were covered with aluminum foil to protect them from light and stored in a refrigerator at 4 °C. Within 1 h, plasma or serum obtained by centrifugation at 1500 *g* (plasma) or 3000 *g* (serum) for 10 min at 4 °C were pooled and aliquots of 100 μl were collected in amber cryovials, covered with aluminum foil and stored at −80 °C. The retinol and tocopherol contents of the pooled serum or plasma samples were quantified using a kit for the HPLC analysis of vitamins A and E in serum provided by Chromsystems and the results were compared with those obtained for lyophilized serum controls.

2.5. Sample preparation

The extraction procedure was carried out in a room protected from direct sunlight and lit by subdued yellow light. Plasma or serum (100 μl) was mixed with 200 μl ethanol–chloroform (3:1, v/v), vortex mixed for 1 min, allowed to stand for 5 min, and mixed for a further 1 min. After centrifugation at 12 000 r.p.m. for 8 min at 4 °C, 200 μl of the clear supernatant was transferred to an amber conical autosampler vial. Vials were capped, mixed by inversion and immediately placed in the sample compartment of the autoinjector, from which 20 μl of the sample, corresponding to 6.7 μl serum or plasma, was injected into the HPLC system for analysis.

2.6. Chromatographic separation

Prior to use, eluent components were degassed by bubbling with helium. The solvent system used was a gradient of solvent A (water) and B (methanol). Vitamins were eluted from 97% B (0–4.5 min) to 100% B (5–13 min) at room temperature. The gradient was then immediately returned to 97% solvent B (13–14 min) and the initial conditions restored in 5 min. The flow-rate was 1 ml/min. A Model 122 fluorometric detector operating at an excitation wavelength of 325 nm and an emission wavelength of 470 nm for vitamin A and at an excitation wavelength of 295 nm and an emission wavelength of 330 nm for vitamin E was used. The spectral bandwidths were 10 nm for both, and the excitation and emission wavelengths and the digital filter w/response speed was 5 s. The detector was programmed to monitor retinol from 0 to 5.0 min and tocopherols from 7. 0 to 13 min. Each vitamin was quantitated on the basis of peak height using the calibration curves previously generated from standard solutions. Each determination was performed in quadruplicate.

2.7. Recovery

In order to establish the recovery of vitamins A and E, three groups of amber Eppendorf tubes (A, B and C) were prepared, and 200 μl of mixed working standard were added to the tubes of groups A and C. The tubes were then flushed with nitrogen to dryness. At this point, 200 μl of water was added to each tube of group A and 200 μl of pooled serum sample to each tube of groups B and C. After mixing, 400 μl of ethanol–chloroform (3:1, v/v) was added for extraction. The mixture was shaken on a vortex mixer for 1 min, allowed to stand for 5 min, and mixed for a further 1 min. After centrifugation (8 min at 12 000 r.p.m. and 4 °C), 400 μl of the clear supernatant was transferred to an amber conical autosampler vial, from which a 20 μl aliquot, corresponding to 6.7 μl of the original volume, was injected into the HPLC system for analysis.

3. Results and discussion

Several HPLC methods have been reported for all-*trans*-retinol and α-tocopherol determination [13–23] in human serum or plasma. Most of them require protein precipitation, liquid–liquid extraction, drying under nitrogen and reconstitution of the sample prior

to analysis. In general, the procedures are quite laborious and carry the risk of artifactual transformation such as isomerization, oxidation and degradation.

In the method reported here, two volumes of an ethanol–chloroform mixture (3:1) are added to serum or plasma samples to produce protein precipitation and lipid extraction, without I.S. addition. Vitamin separation is achieved by reversed-phase chromatography using a two-step gradient elution (97 and 100% methanol in water) with fluorometric detection performed at room temperature (about 25 °C). The chromatographic profile of the fat-soluble vitamins of a representative human serum sample is shown in Fig. 1. It shows a peak containing β-tocopherol and γ-tocopherol (peak 2 with a retention time of 7.3 min) between retinol (peak 1 with a retention time of 3.2 min) and α-tocopherol (peak 3 with a retention time of 8.2 min), while δ-tocopherol (retention time 6.8 min) was not detected. β- and γ-tocopherol were not separated and were quantitated together, which is acceptable because, in humans, β- and γ-tocopherol show a lower specificity than α-tocopherol. The latter is selectively retained in the body and shows preferential interactions with molecular components of cells [12].

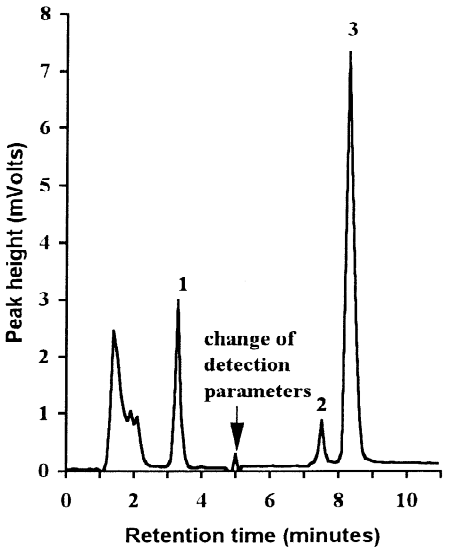

Fig. 1. RP-HPLC analysis of all-*trans*-retinol and tocopherols from normal serum. Twenty microliters of the ethanol–chloroform extract, equivalent to 6.7 μl of the original sample, was injected into the HPLC system and analyzed. Chromatographic conditions, peak names and corresponding retention times are the same as those reported in Fig. 2.

3.1. Statistical parameters for vitamin standard curves

The procedure was first checked using a mixture of standards. The concentrations of the working standard, prepared by diluting a mixture of stock solutions with water–ethanol–chloroform as reported in Section 2.3, and expressed as ng×20 μl^{-1}, correspond to those present in 6.67 μl of the original serum or plasma sample (1, 2, 3, 4 and 8 ng of all-*trans*-retinol, 10, 20, 30, 90 and 180 ng of α-tocopherol, and 1, 3, 6, 8 and 10 ng of γ-tocopherol).

A linear relationship was established between vitamin concentration and peak height. For each concentration, four samples were analyzed and linear regression parameters (estimated intercept b_0; estimated slope b_1) and coefficients of correlation (r) were calculated. The same relationship was also ascertained between vitamin concentration and peak area (data not shown). The following formula can be applied for manual calculation:

$$[x]\ \mu\text{mol/l} = \{[(y - b_0)/b_1] \cdot 150\}/MW$$

where y is the peak height of the analyte, b_0 is the estimates intercept, and b_1 is the estimated slope.

In order to establish the reproducibility of the method, the same run was repeated for a period of 3 weeks, and intra- and inter-day coefficients of variation (C.V.) were calculated. Table 1 shows that the calibration curves exhibited excellent linearity for both vitamin A and E. The correlation coefficients were greater than 0.9980 for the concentration range investigated.

A working standard mixture, i.e. 2 ng of all-*trans*-retinol, 40 ng of α-tocopherol, and 5.9 ng of γ-tocopherol, was used to monitor the sensitivity on a daily time scale. The elution pattern of this working standard mixture is shown in Fig. 2. α-Tocopherol was eluted from the column at 8.20 min, but elution was stopped at 15 min to allow stabilization of the column and detector. The retention times of the vitamins were reproducible (±0.02 min).

Table 1
Linear regression parameters and intra- and inter-day mean coefficients of variation for the HPLC assay of vitamins A and E

Compound	Estimated slope (b_1) (mean±SD)	Estimated intercept (b_0) (mean±SD)	Correlation coefficient (r) (mean±SD)	Intra-day C.V. (%)	Inter-day C.V. (%)
All-*trans*-retinol	0.464±0.05	−0.034±0.004	0.9987	1.64	3.35
α-Tocopherol	0.064±0.004	0.07±0.006	0.9999	1.42	2.90
γ-Tocopherol	0.065±0.004	0.06±0.005	0.9984	1.57	3.10

The intra-day C.V. was calculated from four different concentrations of a standard vitamin mixture. For each concentration, four samples were analysed. The same run was repeated for a period of 3 weeks, and the inter-day C.V. was calculated.

3.2. Extraction of vitamins

Because ~90% of the total plasma vitamin E in humans is associated with the low-density lipoprotein and high-density lipoprotein fractions [25], current protein precipitation procedures with ethanol, methanol, isopropanol or acetonitrile are followed by extraction with organic solvents such as *n*-hexane, petroleum ether, dichloromethane–methanol, chloroform and acetone–chloroform. In our opinion, this procedure can be shortened by organic solvents, which can perform lipid extraction by breaking up the lipoproteins. To this end, we first tested three solvents (ethanol, methanol and isopropanol) as precipitating agents, quantifying the fat-soluble vitamins simultaneously extracted. The best precipitation and extraction conditions were achieved with two volumes of ethanol (Fig. 3). In order to enhance vitamin recovery we also tested ethanol mixed with chloroform in various ratios. The highest vitamin yield was obtained by treating serum or plasma with two volumes of a 3:1 ethanol–chloroform mixture (Fig. 4). The addition of this organic phase led to a finely grained protein precipitate and to complete extraction of vitamins A and E. These extracts,

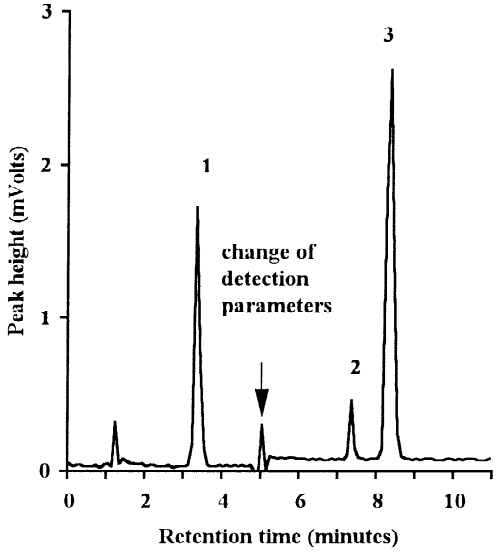

Fig. 2. Chromatogram of the reference working standard mixture. Twenty microliters of ethanol–chloroform (3:1) containing 2 ng of all-*trans*-retinol, 40 ng of α-tocopherol and 5.9 ng of γ-tocopherol was injected and eluted as reported in Section 2.6. Peak 1, all-*trans*-retinol (retention time 3.2 min); peak 2, γ-tocopherol (7.3 min); peak 3, α-tocopherol (8.2 min).

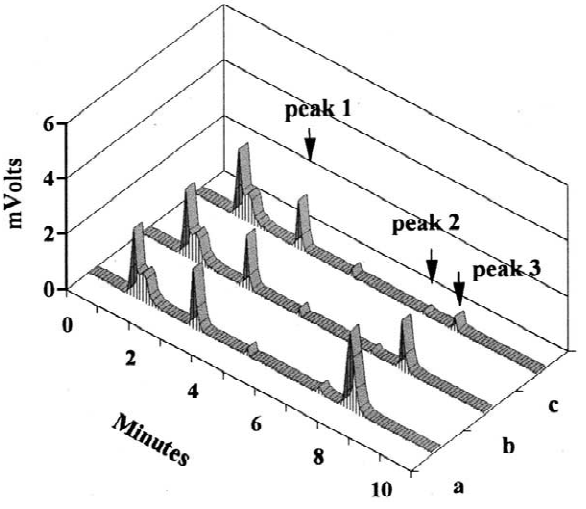

Fig. 3. RP-HPLC analysis of all-*trans*-retinol and tocopherols in serum extracted with ethanol, isopropanol and methanol to optimize protein precipitation and vitamin yields. Pooled serum samples were extracted with two volumes of absolute ethanol (profile a), or isopropanol (profile b), or methanol (profile c). Chromatographic analysis and detection are reported in Section 2.6. Peaks of all-*trans*-retinol and tocopherols are numbered as in Fig. 2.

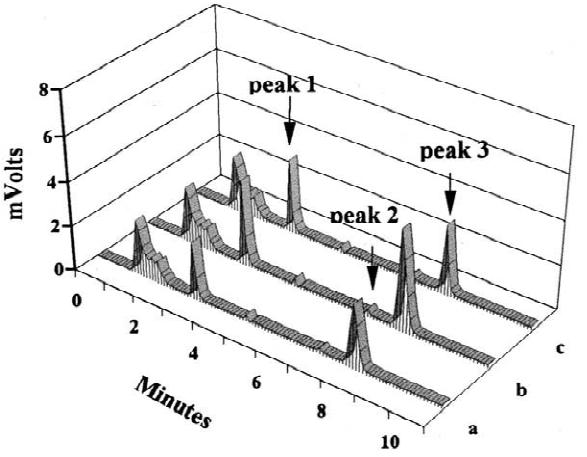

Fig. 4. RP-HPLC analysis of all-*trans*-retinol and tocopherols in serum extracted with ethanol or ethanol–chloroform mixtures to optimize vitamin yields. Pooled serum samples were extracted with two volumes of absolute ethanol (profile a), or with two volumes of a 3:1 ethanol–chloroform mixture (profile b) or with two volumes of a 4:1 ethanol–chloroform mixture (profile c). Chromatographic analysis and detection are reported in Section 2.6. Peaks of all-*trans*-retinol and tocopherols are numbered as in Fig. 2.

stored at 7 °C for up to 48 h in the autoinjector or at 18 °C for 8 h in the dark, did not show any appreciable change in peak height. This allows the analysis of a large series of samples. The extraction procedure performed on pooled samples of both human serum and plasma led to the same results.

3.3. Recovery

The mean recoveries (%) for six independent experiments were: retinol 100.7%, α-tocopherol 98.9% and γ-tocopherol 98.6% (see Table 2).

3.4. Reproducibility of results and validation method

Reproducibility of the method was checked by multiple determinations of a pooled human plasma sample on the same day (intra-batch precision). The following C.V. values were obtained: retinol 2.2% ($n=10$), α-tocopherol 2.4% ($n=10$) and γ-tocopherol 2.8% ($n=10$) (Table 3). Different samples of the same pooled human plasma were used for each assay over a 12-week period on 30 different days to test the day-to-day reproducibility of the method. The inter-batch C.V. values were: retinol 5.1% ($n=20$), α-tocopherol 3.2% ($n=20$) and γ-tocopherol 3.8% ($n=20$) (Table 3).

Table 2
Recovery of retinol, α-tocopherol and γ-tocopherol

Compound	Concentration (mean±SD, $n=6$) (μmol/l)			Recovery[b] (%)
	A[a]	B[a,c]	C[a]	
Retinol	1.0±0.1	1.6±0.1	2.6±0.2	100.8
α-Tocopherol	13.6±0.4	24.1±1.2	37.5±2.3	99.5
γ-Tocopherol	2.1±0.1	2.1±0.1	4.1±0.2	98.6

[a] A, water+working standards; B, serum only; C, serum+working standards.
[b] Recovery = $C \cdot 100/A + B$.
[c] Concentration of retinol, α-tocopherol and γ-tocopherol in pooled serum or plasma samples.

In total, we analysed 120 samples and the concentrations measured ranged from 172 to 900 μg/l (0.60–3.15 μmol/l, mean 1.87 μmol/l) for retinol, and from 3.50 to 20.26 mg/l (8.0–47.6 μmol/l, mean 27.8 μmol/l) for α-tocopherol. For β- and γ-tocopherol the concentrations ranged from non-detectable to 1.3 mg/l (3 μmol/l). The mean serum or plasma concentrations found were in agreement with those reported in the literature for retinol and α-tocopherol [26].

3.5. Quantification limit

The lower limit of quantification, defined as the lowest quantitatively measurable concentration of the different compounds (μmol/l), was calculated with an accuracy of 95% according to Funk et al. [27]; all-*trans*-retinol 0.105 μmol/l (0.70 pmol/6.7 μl of serum or plasma), α-tocopherol 0.696 μmol/l (4.64

Table 3
Intra-day and inter-day precision

Compound	Level[a] (mean±SD, $n=6$) (μmol/l)	Intra-day C.V. (%) ($n=10$)	Inter-day C.V. (%) ($n=20$)
All-*trans*-retinol	1.6±0.1	2.2	5.1
α-Tocopherol	24.1±1.2	2.4	3.2
γ-Tocopherol	2.1±0.1	2.8	3.8

[a] Concentration of retinol, α-tocopherol and γ-tocopherol determined in pooled samples of serum or plasma.

pmol/6.7 μl of serum or plasma), and γ-tocopherol 0.6 μmol/l (4.0 pmol/6.7 μl of serum or plasma).

4. Conclusion

In this paper we report a very simple and rapid procedure for the simultaneous extraction and analysis of retinol and tocopherols from human serum and plasma. Because sample preparation is straightforward, excellent precision is obtained without the use of an internal standard. Another advantage of the present procedure is that only a small volume of plasma or serum is required to perform the analysis (50–100 μl). Thus, it can be used for detecting vitamins in preterm neonate plasma where the concentration of α-tocopherol is approximately one-third of that present in maternal plasma [28]. The simplicity of extraction, the short run time, and the good reproducibility make this procedure ideal for the detection of vitamins A and E in a large series of samples generated by clinical studies.

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