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Note

Simultaneous determination of retinol and α-tocopherol in human serum by high-performance liquid chromatography

ANDRE P. DE LEENHEER*, VEERLE O.R.C. DE BEVERE, MARCEL G.M. DE RUYTER and ALBERT E. CLAEYS

Laboratoria voor Medische Biochemie en Klinische Analyse en voor Analytische Chemie, Faculteit van de Farmaceutische Wetenschappen, Academisch Ziekenhuis, Rijksuniversiteit Gent, De Pintelaan 135, B-9000 Gent (Belgium)

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All-*trans*-retinol is the predominant form of vitamin A in serum (>95%), whereas *d*-α-tocopherol represents 88% of the total vitamin E pool of serum (2% exists in the *d*-β and 10% in the *d*-γ form) [1—3]. The sparing action of vitamin E on the expenditure of vitamin A has been suggested [4—6]. In view of this hypothesis the combined analysis of these two vitamins might prove to be clinically useful.

The simultaneous determination of vitamin A and E in serum has only rarely been performed. Two procedures [7, 8] using fluorimetry have been reported. Both methods are based on the difference in fluorimetric properties of the two molecules.

Chromatographic separation and determination of these two vitamins using thin-layer chromatography (TLC) [9], gel chromatography [10, 11] and high-performance liquid chromatography (HPLC) [12] have been described. However, these methods have not been applied to biological samples. Separate HPLC assays for vitamin A [13] and E [14] in serum were recently reported by us.

We now propose an HPLC method for the simultaneous determination of retinol and α-tocopherol in serum (or plasma).

*To whom correspondence should be addressed at the Laboratoria voor Medische Biochemie en Klinische Analyse.

EXPERIMENTAL

Materials

Methanol, ethanol, *n*-hexane were all analytical-grade reagents from E. Merck (Darmstadt, G.F.R.) and used without further purification.

All-*trans*-retinol was of crystalline purity from Sigma (St. Louis, Mo., U.S.A.); *d*-α- and *d*-β-tocopherol were purchased from Eastman-Kodak (Rochester, N.Y., U.S.A.) and *d*-γ-tocopherol was obtained from Supelco (Bellefonte, Pa., U.S.A.).

The internal standard *dl*-tocol was purchased from Koch-Light (Colnbrook, Great Britain).

High-performance liquid chromatography

We used a Hewlett–Packard 1084A liquid chromatograph equipped with a Pye Unicam LC3 variable-wavelength detector.

The chromatographic support RSIL C_{18}, a 10-μm heavily loaded octadecyl bonded phase (18% organic material) and the column tubing (25 cm × 4.6 mm I.D., Lichroma SS) were obtained from RSL (St. Martens-Latem, Belgium). The column was packed by the slurry technique under following conditions: slurry liquid, tetrachloromethane (analytical grade, E. Merck); slurry concentration, 8% (w/v); pump, Varian 8500; packing pressure, 210 bar; pressurising liquid, methanol. At optimum flow-rate (0.25 ml/min), a column efficiency of 11,000 plates (h = 2.3) was obtained for α-tocopherol.

Elution was performed by methanol at a flow-rate of 2 ml/min (pressure = 96 bar). The oven temperature was set at 40° and the column effluent was monitored at 292 nm.

Sample preparation

In a PTFE-capped centrifuge tube (100 × 10 mm), 10 μl of a tocol internal standard solution (0.1 μg/μl) in ethanol and 100 μl of pure ethanol were added to a serum (or plasma) sample of 100 μl.

After mixing, the sample was extracted with 1 ml of *n*-hexane by interrupted mixing on a Vortex-type mixer for 4 min. After centrifugation (10 min, 1500 *g*) the organic layer was evaporated at 40° in a gentle stream of nitrogen. The residue was dissolved in 50 μl of methanol and injected on top of the RP-18 column.

Quantification

Known amounts of vitamins A and E covering the range of 125–1000 μg/l and 5–25 mg/l respectively, were added to samples of a serum pool. Calibration curves were made by plotting peak height ratios ($h_{retinol}/h_{i.s.}$; $h_{\alpha\text{-tocopherol}}/h_{i.s.}$) against vitamin A and E concentrations.

RESULTS

Fig. 1 shows a typical chromatogram obtained from a serum extract to which a known amount of tocol as internal standard had been added. Retention characteristics are given in Table I. The β- and γ-tocopherol isomers elute

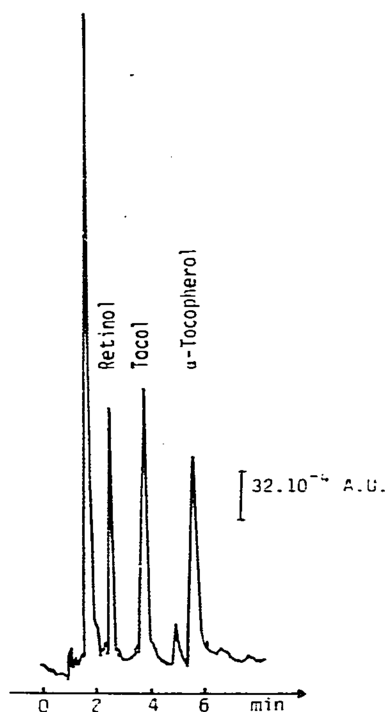

Fig. 1. High-performance liquid chromatogram of a serum extract. Column: 25 cm × 4.6 mm I.D., packed with 10-μm RSIL C_{18}. Eluent: methanol. Flow-rate: 2.0 ml/min. Oven temperature: 40°. Detection: 292 nm.

TABLE I

CAPACITY RATIOS (k') OF CHROMATOGRAPHIC PEAKS

Compound	k'
All-*trans*-retinol	1.06
Tocol (internal standard)	2.10
β- and α-Tocopherol*	3.01
α-Tocopherol	3.54

*Composite peak.

as one peak. The total elution time of the two vitamins and the internal standard takes less than 6 min.

A linear relationship between peak height ratios (peak height of retinol or α-tocopherol:peak height of tocol) and retinol or α-tocopherol concentrations was found. These calibration curves ($y = 0.0012\,x + 0.9398$, $r = 0.9991$ and $y = 0.0671\,x + 0.6748$, $r = 0.9994$, respectively) are used after subtraction of the intercepts, which represent the endogenous levels of the vitamins in the serum pool. Concentrations in unknown serum samples are then easily determined after calculation of the peak height ratios.

The mean extraction recoveries from spiked serum, as given in Table II, were 96.8 and 95.5% for retinol and α-tocopherol, respectively.

In serum the lower detection limit for retinol was estimated at 60 μg/l, whereas a value of 0.6 mg/l was found for α-tocopherol. A within-day precision (coefficient of variation, C.V.) of 2.47% and 1.62% was obtained for retinol and for α-tocopherol, respectively, analyzing 10 samples of a normal serum pool ($\bar{x}$ = 674 μg/l, S.D. = 16.6 μg/l for retinol; $\bar{x}$ = 11.27 mg/l, S.D. = 0.18 mg/l for α-tocopherol). The day-to-day precision (C.V.) as measured over a period of 20 days was 4.9% for retinol and 3.2% for α-tocopherol, at the respective concentration levels of 635 μg/l and 10.8 mg/l. For these experiments, serum samples were stored at −18° under a nitrogen atmosphere.

TABLE II

EXTRACTION RECOVERY

Compound	$\bar{x}$ (%)	S.D. (%)	C.V. (%)	*n*	Range (mg/l)
Retinol	96.8	3.0	3.1	4	0.25–1.00
α-Tocopherol	95.5	3.7	3.9	5	5 –25

Peaks obtained from a serum extract are identified on the basis of retention times. The specificity of the method was also checked by determining the $\lambda_{max.}$ of the peaks eluting at the retention times of retinol, α, β, γ-tocopherol and tocol. Therefore, aliquots of an extract of a 1-ml serum sample were run at different wavelength settings and peak heights of compounds of interest were plotted vs. wavelength. The $\lambda_{max.}$ we found ($\lambda_{max.}$ (retinol) = 330 nm, $\lambda_{max.}$ (tocopherols) = 295 nm) agreed with values reported in the literature [15, 16]. In addition, for the tocopherols the specificity of the procedure was further verified by gas–liquid chromatography and gas chromatography–mass spectrometry [14].

As a test of the applicability of the method, serum from a series of 25 human donors was analyzed for retinol and α-tocopherol. Data are given in Table III.

DISCUSSION

This simultaneous determination of vitamins A and E in serum by HPLC, provides high specificity due to a chromatography column with a high separation capacity. The addition of a known amount of tocol as an internal standard before the extraction step, compensates for possible losses, caused by evaporation or spilling, and improves the precision of the method. Tocol was chosen as an internal standard on the basis of its retention characteristics (it elutes between the two vitamins assayed), commercial availability and structural analogy to the component with the lowest extinction coefficient i.e. α-tocopherol. The major absorption band of α-tocopherol and tocol is relatively

TABLE III

SERUM RETINOL AND α-TOCOPHEROL CONCENTRATIONS FOR SOME ADULT HUMANS

Age (years)	Sex	Retinol (μg/l)	α-Tocopherol (mg/l)
69	F	625	11.4
72	F	835	9.5
64	M	1000	7.9
32	F	505	5.0
50	M	980	7.3
15	M	635	6.2
65	F	745	6.0
62	F	775	9.4
70	M	710	6.4
77	F	790	9.4
31	F	565	7.4
45	M	610	8.2
51	F	760	15.3
39	M	775	9.8
83	F	495	10.6
54	M	270	7.7
77	M	715	18.5
67	M	230	8.8
57	M	830	19.8
56	M	570	11.3
68	F	565	10.5
56	M	270	5.5
65	M	370	6.2
75	M	342	10.5
71	F	403	6.6

sharp and occurs at 292 nm (ϵ = 3500 and 3900 $l \cdot mol^{-1} \cdot cm^{-1}$, respectively). This compound has practically no residual absorbance at 325 nm, the $\lambda_{max.}$ of retinol (ϵ = 52600 $l \cdot mol^{-1} \cdot cm^{-1}$). However, retinol still absorbs significantly at 292 nm, the $\lambda_{max.}$ of α-tocopherol. We, therefore, monitored the column effluent at 292 nm, the wavelength of the component with the weakest absorbance.

The method proposed is sensitive as only 100 μl of serum (or plasma) are needed. The overall procedure is simple and the total analysis time is relatively short (less than 30 min including sample preparation, extraction and chromatography). Compared to the fluorimetric method of Hansen and Warwick [8], our method proved to be more precise: the C.V. reported for the fluorimetric technique was 7% against 3% for our method. The specificity of our retinol determination should also be better, because it is well-documented that other (fluorescent) co-extracted compounds interfere with the fluorimetric method. To overcome this problem, Thompson et al. [7] introduced a correction formula which compensates specifically for the presence of phytofluene. However, because fluorescence must be measured at two emission and three excita-

tion wavelengths, their technique appears more complicated. On the other hand the HPLC method uses only one wavelength (292 nm), improving the reliability of the measurement, and no tedious calculations have to be made.

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