CHROMBIO. 3140

## Note

# Simultaneous measurement of retinol and α-tocopherol in human serum by high-performance liquid chromatography with ultraviolet detection

DOLORES CUESTA SANZ and MARIO CASTRO SANTA-CRUZ*

*Departamento de Investigación, Servicio de Dietética and Servicio de Bioquímica, Centro Especial Ramón y Cajal, Ctra. de Colmenar, Km 9, Madrid 34 (Spain)*

Because of the increasing use of vitamins in clinical practice, their estimation in plasma is becoming common in clinical laboratories. We propose a method for simultaneous evaluation of retinol and *dl*-α-tocopherol in serum or plasma by high-performance liquid chromatography (HPLC) with UV detection. Our assay has several advantages over previously published methods [1—4] with respect to sensitivity, specificity and simplicity of use. This makes it a useful tool for investigations in clinical and analytical laboratories.

EXPERIMENTAL

*Reagents and apparatus*
Synthetic and crystallized *trans*-retinol (ca. 40—50%), *dl*-α-tocopherol and synthetic and crystallized *trans*-retinyl acetate were obtained from Sigma (St. Louis, MO, U.S.A.). Methanol, ethanol and *n*-hexane for HPLC were from Scharlau (F.E.R.O.S.A., Spain) and analytical-grade potassium biphosphate and sodium hydroxide from Merck-Igoda (Spain).
Chromatography was carried out using a Waters Chromatograph composed of a Model 510 pump, U6K injector, Model 440 UV detector with filters for 280 and 340 nm and a detection sensitivity of 0.01 a.u.f.s., Data Module 730 integrator, P/N 080040 precolumn Module with an RCSS $C_{18}$ cartridge and a Nucleosil 5 $C_{18}$ column (120 mm × 3 mm I.D.) from Macherey-Nagel (F.I.O.S.A., Spain).

*Standards*
A stock solution of retinol and *dl*-α-tocopherol (0.5 and 7 mg/ml, respective-

ly) was prepared in ethanol and a stock solution of internal standard, retinyl acetate (1.2 mg/ml), was prepared in ethanol. The two reference solutions were stored separately at $-24°$C.

On the day of analysis of retinol and $dl$-$\alpha$-tocopherol, the stock solution was diluted 1:100 with ethanol. The retinyl acetate (internal standard) stock standard solution was diluted 1:500 with ethanol. Concentrations of the working standard solution were determined spectrophotometrically by using the following extinction coefficients ($E_{1\,cm}^{1\,\%}$): retinol, 1830 at 325 nm [5] and 1006 at 294 nm (our value); $dl$-$\alpha$-tocopherol, 71 at 294 nm [6].

*Sample preparation, chromatography and quantitation*

Pyrex test tubes (100 × 10 mm) were used for the extraction procedure. To 200 $\mu$l of serum or plasma were added 50 $\mu$l of internal standard, 200 $\mu$l of ethanol and 200 $\mu$l of methanol. After thorough vortexing for 5 s, the mixture was extracted twice with 1 ml of $n$-hexane by vortexing for 60 s, and then centrifuged at 1000 $g$ for 5 min. The hexane extracts were pooled and evaporated under nitrogen. The dry residue was redissolved with 200 $\mu$l of methanol and 50-$\mu$l aliquots of this solution were injected into the chromatograph.

The standards were processed in the same way by using 200 $\mu$l of 5 m$M$ potassium biphosphate—sodium hydroxide (pH 7.5) instead of serum, 50 $\mu$l of internal working standard, 150 $\mu$l of ethanol and 200 $\mu$l of methanol.

Chromatography was carried out using methanol—water (95:5) as eluent at a flow-rate of 2.0 ml/min (pressure: 186 bar). Detection was at 340 nm for the first 3 min and at 280 nm thereafter (3 min).

To quantify retinol and $dl$-$\alpha$-tocopherol in the samples, the working standard was always analysed along with the samples and peak-area ratios were used for calculations following the internal standard method.

RESULTS

*Calibration curves*

Calibration curves obtained from standard solutions of increasing concentrations of retinol and $dl$-$\alpha$-tocopherol (3—198 $\mu$g per 100 ml and 114—7294 $\mu$g per 100 ml, respectively) gave the following linear regression equations and coefficients of correlation: retinol, $y = -0.044 + 1.206 \cdot 10^{-2}x$, $r = 0.9998$; $dl$-$\alpha$-tocopherol, $y = -0.073 + 4.285 \cdot 10^{-4}x$, $r = 0.9996$.

*Precision*

The within-day and between-day coefficients of variation (C.V.) are shown in Table I. Coefficients of variation for either parameter were of the order of 3% (Table I).

*Recovery*

The recovery experiment was carried out by adding increasing amounts of each compound under study to a serum sample, which was treated identically to the samples. Average recoveries were 103.1% for retinol and 90.2% for $dl$-$\alpha$-tocopherol. Fig. 1 shows two representative correlations between theoretical

TABLE I

WITHIN-DAY AND BETWEEN-DAY PRECISION

| Compound | Concentration (mean ± S.D.) (µg per 100 ml) | $n$ | Coefficient of variation (%) |
|---|---|---|---|
| *Within-day* | | | |
| Retinol | 47.4 ± 2.0 | 5 | 1.85 |
| *dl-α*-Tocopherol | 1218 ± 77 | 5 | 2.84 |
| | | | |
| *Between-day* | | | |
| Retinol | 50.3 ± 2.4 | 6 | 2.40 |
| *dl-α*-Tocopherol | 1289 ± 82 | 6 | 2.60 |

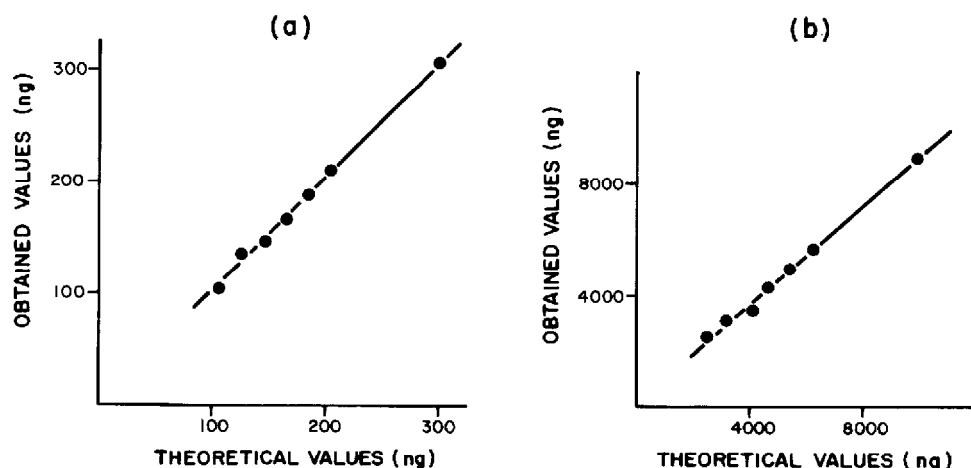

Fig. 1. Linear correlation between theoretical and experimental values of retinol (a) and *dl-α*-tocopherol (b) assayed in serum recovery determination.

and actual values of retinol (a) and *dl-α*-tocopherol (b). It is evident that in both cases a highly significant linear correlation was obtained.

*Detection limits*

The correlation coefficients were high and the detection limits of the method were 3 µg per 100 ml for retinol and 100 µg per 100 ml for *dl-α*-tocopherol.

*Interferences*

All the samples processed for retinol and *dl-α*-tocopherol quantitation with the present method were obtained from hospital in-patients under different therapies, both medical and surgical. An interference was detected in samples from a group of psychiatric patients under treatment with different drugs, haloperidol intake being common to all of them. In samples from these patients, the chromatogram showed a peak incompletely resolved from that of α-tocopherol, producing an underestimation of calculated vitamin E of about 10% of the actual value. No other interference was detected.

TABLE II

NORMAL VALUES OF RETINOL AND *dl*-α-TOCOPHEROL

Number of volunteers are given in parentheses. Retinol: $p < 0.05$; *dl*-α-tocopherol: non-significant.

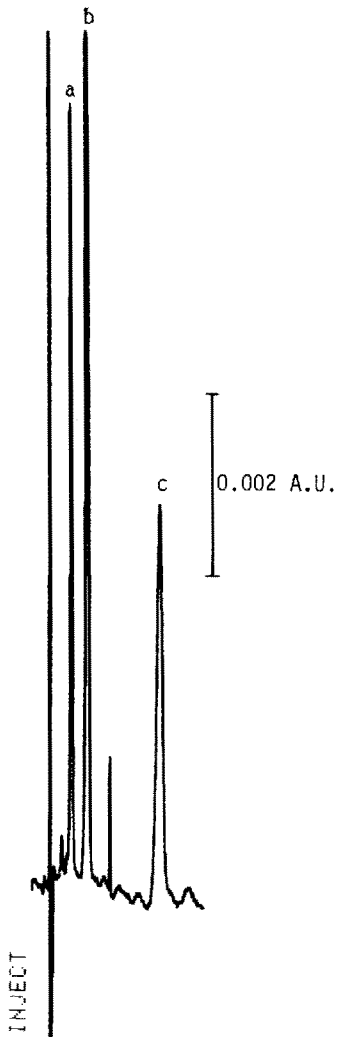| Compound | Concentration (mean ± S.D.) ($\mu$g per 100 ml) | |
|---|---|---|
| | Men | Women |
| Retinol | 63 ± 10 (22) | 50 ± 12 (32) |
| *dl*-α-Tocopherol | 1181 ± 225 (22) | 1176 ± 275 (32) |



Fig. 2. Representative chromatogram of a serum sample processed as indicated in the text. Peaks: a = retinol; b = retinyl acetate; c = α-tocopherol.

*Normal values*

Serum concentrations of retinol and *dl-α*-tocopherol obtained from apparently healthy adults (32 women and 22 men) are shown in Table II. Retinol values were slightly but significantly lower in women than in men, whereas no difference was found in *dl-α*-tocopherol levels.

DISCUSSION

Although various methods for the simultaneous determination of vitamins A and E have been published [1–4], we consider the proposed method of interest not only because of its selectivity and sensitivity but also because of its simplicity and type of equipment used.

Fig. 2 represents a chromatogram of a serum sample. All peaks were well resolved with minimal analysis time (6 min). The short analysis time and the absence of late eluting compounds enables processing of a large number of samples.

By comparing present results with those obtained by other authors [1– 3] it is evident that this method affords higher sensitivity and selectivity for vitamin A. The difference may reside in the wavelength (340 nm) used in this work, which is closer to the absortion maximum than that used by others (290 nm).

Finally, the results of precision shown in Table I demonstrate the reproducibility of the present method.

ACKNOWLEDGEMENTS

REFERENCES

1 A.P. De Leenheer, V.O.R.C. De Bevere, M.G.M. De Ruyter and A.E. Claeys, J. Chromatogr., 162 (1979) 408—413.
2 W.J. Driskell, J.W. Neese, C.C. Bryant and M.M. Bashor, J. Chromatogr., 231 (1982) 439—444.
3 P. Söderhjelm and B. Andersson, J. Sci. Food Agric., 29 (1978) 697—702.
4 A.T. Rhys Williams, J. Chromatogr., 341 (1985) 198—201.
5 R. Strobecker and H.M. Henning, Vitamin Assay, Verlag Chemie, 1st ed., 2nd printing, 1966, p. 34.
6 The Merck Index, Merck and Co., Rahway, NJ, 1976, p. 1920.