Journal of Chromatography, 341 (1985) 198—201
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2547

Note

Simultaneous determination of serum vitamin A and E by liquid chromatography with fluorescence detection

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(First received October 22nd, 1984; revised manuscript received December 28th, 1984)

Several papers have been published [1—9] on the separate determination of serum/plasma vitamins A and E; relatively few have described the simultaneous determination. Manual, fluorimetric methods based upon the difference in fluorescence characteristics of the two vitamins have been reported [10, 11]. Two procedures for the simultaneous determination have been described using reversed-phase liquid chromatography and UV absorption detection with the wavelength set at 292 nm [12] or at 250 nm [13]. As the absorption spectra (and fluorescence emission) of the vitamins differ considerably [14], monitoring at one wavelength is a compromise. In this paper is described the use of a fluorescence detector which automatically changes the excitation and emission wavelengths with respect to retention time so optimising the selectivity and sensitivity of the analysis.

Normal-phase chromatography is used both for providing compatibility with the solvent extraction procedure and for increasing the sensitivity compared with the high-polarity solvents used in reversed-phase chromatography.

EXPERIMENTAL

Reagents

All-*trans*-retinol and *d*-α-tocopherol were obtained from Sigma (St. Louis, MO, U.S.A.). *n*-Hexane special for liquid chromatography was obtained from Fisons (Loughborough, U.K.), and ethanol, 99.7—100% (AnalaR) was obtained from BDH (Poole, U.K.). Stock standards were made up in ethanol and diluted in ethanol in the range 6.0—60 μmol l^{-1} for *d*-α-tocopherol and 0.4—4.0 μmol l^{-1} for *d*-1-retinol.

0378-4347/85/$03.30

Apparatus

Chromatography was carried out using a Perkin-Elmer Series 4 pump and a Rheodyne 7125 injector fitted with a 20-μl loop. The fluorescence detector was a Perkin-Elmer Model LS-4. The silica column was an HS-3 100 × 4.6 mm column with 3-μm silica. Elution was performed with *n*-hexane—ethanol (99:1) at 2 ml min^{-1} at ambient temperature. The Model LS-4 was programmed to monitor first at 295 nm excitation, 390 nm emission and after 2.5 min to change to 325 nm excitation, 480 nm emission.

Sample preparation

In a PTFE-capped 100 × 10 mm centrifuge tube 200 μl ethanol were added to 200 μl of serum sample. After vortex-mixing to ensure effective precipitation of the proteins, 200 μl of water were added followed by 200 μl of the mobile phase. The samples were further vortex-mixed for 1 min and then centrifuged at 4500 *g* for 3 min. A 20-μl aliquot of the clear top organic phase was injected directly onto the silica column. Sample recovery was tested by using spiked serum samples, substituting the 200 μl of ethanol with 200 μl of the standards.

Standard calibration

A similar procedure to that of the sample preparation was used substituting 200 μl of water for the serum sample and 200 μl of the ethanolic standards for the 200 μl of ethanol. Calibration curves were made by plotting peak height against vitamin A and E concentrations.

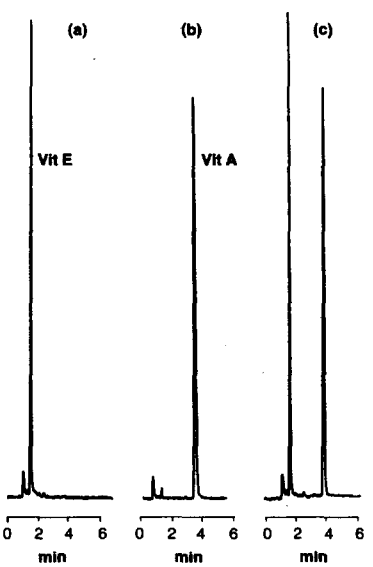

Fig. 1. Liquid chromatograms of serum extracts using procedures described in the text. (a) With the detector set at excitation 295 nm, emission 390 nm; (b) with the detector set at excitation 325 nm, emission 480 nm; (c) with the detector set initially at excitation 295 nm, emission 390 nm and programmed to change to excitation 325 nm, emission 480 nm 2.5 min after injection of the sample. The serum vitamin concentrations were 31.1 and 1.52 μmol l^{-1} for *d*-α-tocopherol and *d*-1-retinol, respectively.

RESULTS

Fig. 1 shows a typical chromatogram obtained from a serum extract with capacity factors (k') of 0.6 and 3.0 for *d*-α-tocopherol and *d*-1-retinol, respectively. A linear relationship between peak height and concentration was observed over the working range. The coefficients of the equations (a = intercept, b = slope and r = regression coefficient) were as follows: *d*-α-tocopherol, $a = 0.0433$, $b = 0.295$, $r = 0.991$; *d*-1-retinol, $a = 0.0478$, $b = 0.3996$, $r = 0.998$. The mean extraction recoveries from four spiked samples were 96.9% for *d*-1-retinol and 98.5% for *d*-α-tocopherol with coefficients of variation of 2.0% and 4.5%, respectively. Concentrations of *d*-α-tocopherol and *d*-1-retinol in various serum samples agreed well with those found by previous studies (Table I). Values ranged from 24.31 to 63.99 μmol l^{-1} for *d*-α-tocopherol and 1.88 to 3.26 μmol l^{-1} for *d*-1-retinol. The coefficient of variation analysing ten samples of a serum pool was 4.90% for *d*-α-tocopherol and 2.02% for *d*-1-retinol at mean values of 31.1 and 1.52 μmol l^{-1}, respectively. The sensitivity of detection using 200-μl samples and 20-μl injection volume was 0.02 μmol l^{-1} for *d*-1-retinol and 0.37 μmol l^{-1} for *d*-α-tocopherol using the basis of a signal twice the noise level.

TABLE I

d-α-TOCOPHEROL AND *d*-1-RETINOL LEVELS IN DIFFERENT SERUM (n = 10)

d-α-Tocopherol (μmol l^{-1})	*d*-1-Retinol (μmol l^{-1})
31.1 (24.31—63.99)	2.08 (1.88—3.26)

DISCUSSION

The choice of normal-phase chromatography was based on two main criteria. These were (a) to obtain the most sensitive assay and (b) to maintain compatability between the extraction procedure and the chromatography. Many organic compounds exhibit quite large changes in fluorescence intensity when measured in solvents of varying polarity [15]. Both *d*-α-tocopherol and *d*-1-retinol show this effect with a five- to six-fold decrease in intensity on going from *n*-hexane to acetonitrile—water (50:50). Repeated injections of *n*-hexane extracts onto reversed-phase columns result in a deterioration of the chromatography. This is probably due to the build-up of lipids and other fat-soluble products on the column. The combination of fluorescence detection (for better sensitivity) and normal-phase chromatography removes the necessity of evaporating to dryness the organic extract and its uptake in a different mobile phase. Ethanol was used as a precipitant in preference to methanol as it gave a better recovery for *d*-α-tocopherol.

The mobile phase composition chosen gave a good separation of the two vitamins in the shortest convenient time. Although peaks could be distinguished between the unretained peak and that of the *d*-α-tocopherol, a lower solvent polarity would ensure absolute separation. However, lowering the polarity significantly increases the retention of the *d*-1-retinol. As the

procedure gave a reasonable degree of precision no internal standard has been used. Previously published methods have used retinyl esters as internal standards. However, these are not suitable for normal-phase chromatography as they would elute in the void volume. *d*-1-Tocol would appear to be useful as an internal standard [12] as it elutes between the two vitamins. This or any other suitable internal standard would improve the precision of the assay as would standardization using serum samples.

The examination of the fluorescence characteristics of the two vitamins shows that they are widely different. *d*-α-Tocopherol has an excitation and emission maximum of 295 and 330 nm, respectively, whereas, those of *d*-1-retinol are 325 and 480 nm, respectively. However, for serum samples containing mean values of both vitamins, an emission wavelength of 390 nm for the *d*-α-tocopherol gives peaks of comparable intensities. If the correct emission wavelength is used the *d*-α-tocopherol peak would be 25-fold more intense than that of *d*-1-retinol.

The effect of automatically changing the excitation and emission wavelengths during chromatography can be seen in Fig. 1. The first two chromatograms are of a serum sample monitored at the appropriate wavelengths of each vitamin. The third chromatogram is of the same sample but with the detection initially at 295 nm excitation, 390 nm emission and then automatically changed after 2.5 min to 325 nm excitation, 480 nm emission.

The sensitivity of the method is such that the serum sample could be reduced from 200 μl to 25—50 μl, but was kept at 200 μl for ease of handling. The overall procedure of extraction and chromatography is relatively simple and the optimization of detection through wavelength programming reduces the assay time to less than 10 min per sample.

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