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Note

Measurement of vitamin A and vitamin E in human serum by high-performance liquid chromatography

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In the past few years, high-performance liquid chromatography (HPLC) has begun to be used for measurement of metabolites important in nutrition. Among those most amenable to HPLC quantitation are vitamin A and vitamin E, because they are present in serum in high enough concentration to be detected easily by their ultraviolet absorption and because there appear to be no substances in serum that interfere in the HPLC assay. Several procedures for measuring vitamin A and vitamin E separately by HPLC have been published [1—5]. Recently, Bieri et al. [6] and De Leenheer et al. [7] have described HPLC procedures which allow the simultaneous determination of vitamin A and vitamin E from a common serum extract. This paper describes another HPLC method for the simultaneous measurement of vitamin A and vitamin E. A major difference between the three approaches is in the choice and number of internal standards. The procedure of Bieri et al. [6] employs two internal standards, retinyl acetate and tocopherol acetate. The method described in this paper, developed for routine high-volume service use, utilizes only retinyl acetate, which greatly decreases the run time. De Leenheer et al. [7] also use only one internal standard, but it is tocol, a synthetic analogue of vitamin E. The procedure is applied to the determination of the stability of vitamins A and E in serum samples. The HPLC procedure and the older trifluoroacetic acid colorimetric assay for vitamin A [8], which has been used in this laboratory in connection with the Health and Nutrition Evaluation Survey (HANES), are compared to determine the degree of correlation between the two methods.

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EXPERIMENTAL

Materials

All-*trans*-retinol and all-*trans*-retinyl acetate, both in a pure crystalline form, were from Sigma (St. Louis, MO, U.S.A.) and *d*-alpha-tocopherol was from ICN (Cleveland, OH, U.S.A.). Methanol and *n*-hexane were HPLC grade from Fisher Scientific (Atlanta, GA, U.S.A.) and used without further purification. The absolute, undenatured alcohol was from National Distillers and Chemical Corporation (New York, NY, U.S.A.).

A normal human serum pool was established specifically for the vitamins A and E stability studies. Human serum samples used in the HPLC procedure versus colorimetric vitamin A procedure comparison were from HANES survey.

High-performance liquid chromatography

The HPLC pump was an Altex 110A (Berkeley, CA, U.S.A.) equipped with a pulse dampener. The injector was an Altex 210 with a 50-μl loop. Injection was with a 50-μl syringe from Hamilton Company (Reno, NV, U.S.A.). A Waters Assoc. (Milford, MA, U.S.A.) Model 450 detector and a Beckman (Fullerton, CA, U.S.A.) 10-in. recorder were used. The column was a 25 cm × 3.9 mm prepacked Waters μBondapack C_{18} (10 μm particle size). A guard column (Waters), 22 × 3 mm, packed with Waters C_{18} Corasil was attached on-line before the main column. Elution was performed by methanol—water (96:4) at a flow-rate of 2 ml/min (pressure of 45 bar). The column effluent was monitored at 290 nm.

Sample preparation

Serum (100 μl) was pipetted into a 75 × 10 mm test tube. To the serum was added 100 μl ethanol containing a known amount of retinyl acetate in the range of 75 to 100 μg/dl. The tube was vortexed for 5 sec. Hexane (200 μl) was added and the mixture vortexed for 30 sec (the tube was bounced as it was being vortexed to assure thorough mixing of the two layers.) Centrifugation was carried out for 1 min at 1000 *g*. A Pasteur pipet was used to remove about 150 μl of the top layer, which was transferred to another 75 × 10 mm test tube. Tubes were placed in a room temperature water bath, and the hexane was evaporated with a stream of nitrogen. The residue was redissolved in 100 μl ethanol; 50 μl of the solution was injected for chromatography.

Quantitation

Retinol and alpha-tocopherol were quantitated from a standard curve of peak height ratios. To prepare a standard curve, a constant amount of retinyl acetate was combined with five different concentrations of retinol in a range of 20—100 μg/dl and with five different concentrations of alpha-tocopherol in a range of 0.4—2.4 mg/dl. The solution of retinol, retinyl acetate, and alpha-tocopherol was chromatographed and the peak height ratios recorded. A linear relationship between peak height ratios (peak height of retinol or alpha-tocopherol: peak height of retinyl acetate) and concentration ratios (concentration of retinol or alpha-tocopherol: retinyl acetate) was found. The equations for

retinol and alpha-tocopherol were, respectively: $y = 1.61x$ and $y = 0.068x$, where y = peak ratios and x = concentration ratios.

RESULTS

A typical chromatogram of a serum extract containing retinyl acetate as an internal standard is shown in Fig. 1. Each of the peaks of interest was well separated from the others. The total time for the assay with a flow-rate of 2 ml/min was about 6 min. The speed of elution was limited to about 4 ml/min flow-rate because of a deterioration of resolution at higher flow-rates. Beta- and gamma-tocopherol eluted together as one peak, immediately before the alpha-tocopherol peak.

Long-term and within-day precision were excellent. In Table I are shown the results of a precision study in which the same serum sample was assayed fourteen times during one day and sixteen times over two months. The within-day coefficients of variation (C.V.) were 3.6% for retinol and 4.5% for alpha-tocopherol with means of 76.6 μg/dl and 747.5 μg/dl, respectively. The long-term overall coefficients of variation were 3.6% for retinol and 4.2% for alpha-tocopherol with means of 76.8 μg/dl and 735 μg/dl, respectively. Efficiency of recovery of known amounts of retinol and alpha-tocopherol added to serum was 97 ± 2% for retinol and 97 ± 2.5% for alpha-tocopherol (means ± S.E. for eight trials each). In serum, the lower detection limit was estimated to be 10 μg/dl for retinol and 80 μg/dl for alpha-tocopherol.

The standards (retinol, alpha-tocopherol, and retinyl acetate) were stable for

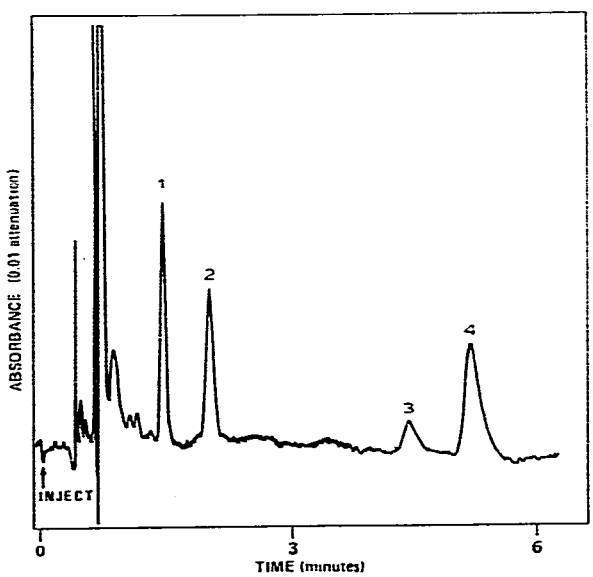

Fig. 1. HPLC chromatogram of a serum extract. Peaks: 1 = retinol; 2 = retinyl acetate; 3 = beta- and gamma-tocopherol; 4 = alpha-tocopherol. Column: 25 cm × 3.9 mm packed with Waters μBondapak C_{18} (10 μm particle size), eluent: methanol—water (96:4) flow-rate: 2.0 ml/min, detector: 290 nm.

TABLE I

SHORT AND LONG-TERM PRECISION OF THE HPLC PROCEDURE FOR RETINOL AND ALPHA-TOCOPHEROL

	Retinol (short-term)* (μg/dl)	Retinol (long-term)** (μg/dl)	Alpha-tocopherol (short-term)* (μg/dl)	Alpha-tocopherol (long-term)** (μg/dl)
	80	79	710	790
	76	80	790	700
	75	71	770	700
	73	77	710	710
	79	76	705	740
	78	80	730	760
	78	74	785	700
	73	74	760	765
	71	76	785	780
	79	73	780	705
	80	81	710	725
	78	77	700	780
	78	75	775	720
	74	81	755	750
		75		720
		80		715
$\bar{X}$	76.6	76.8	747.5	735
S.D.	2.8	2.8	33.5	30.6
C.V. (%)	3.6	3.6	4.5	4.2

*Simple assays, each taken through the entire procedure including extraction, performed over one day. Results listed in chronological order of analysis.

**Daily mean of multiple analyses, each taken through the entire procedure including extraction, performed on sixteen different days over a period of two months. Results are listed in chronological order of assay.

at least two weeks in absolute ethanol at −20°C. After about two weeks of storage a slight shoulder would occasionally appear on the retinyl acetate peak. Retinol and alpha-tocopherol were stable almost indefinitely in absolute ethanol at −20°C. Serum extracts stored in absolute ethanol at −20°C are stable for at least two weeks. This stability permits the convenience of performing the extractions on one day and performing the chromatography on a later day. The lifetime of the HPLC column used for these assays was very long: Over 700 runs were made on the same column with no serious decrease in resolution. The resin in the guard column was changed once during this time.

The HPLC procedure and the trifluoroacetic acid colorimetric procedure were compared for retinol. Three hundred individual serum samples that had been assayed colorimetrically in the HANES study were used. Fig. 2 shows a regression line plot comparing the two methods. The mean for the colorimetric assay was 34.56 μg/dl and the mean for the HPLC method was 32.00 μg/dl. The slope and correlation coefficient were 0.997 and 0.973, respectively. The only obvious outliers were the six samples that show much higher values in the colorimetric assay than the HPLC assay (see Fig. 2). It would be speculation to try to explain the cause of the outliers; but it should be pointed out that the

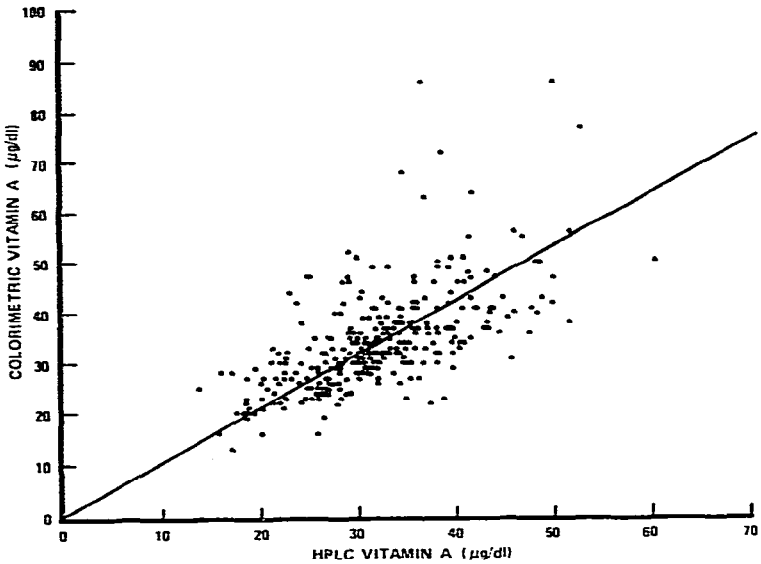

Fig. 2. Regression line plot, utilizing error in both variables model [9], comparing the vitamin A HPLC assay and the TFA colorimetric assay for 300 individual serum samples. The 95% confidence range for the intercept was 0.83—7.6 μg/dl; therefore, regression was forced through zero.

accuracy of the colorimetric assay depends on the presence of only normal carotenoids in serum, i.e., carotenoids that absorb maximally near 450 nm. The contribution of the normal carotenoids to the colorimetric reaction can be cancelled out by subtracting a factor derived from the absorbance of the extract at 450 nm. Although the great majority of carotenoids are normal, there are some naturally occurring short-chain carotenoids which absorb hardly at all at 450 nm [10]. The presence of these would give a falsely high value for retinol.

The question of the stability of vitamin A and vitamin E in serum under various conditions of storage was addressed using the HPLC technique. Samples from a single-donor human serum pool established expressly for the stability study were stored at 25°C, (1 day), 4°C (4 weeks), —20°C (16 months) and —70°C (16 months). Samples were assayed at intervals during these periods of storage. Vitamins A and E in serum were found to be stable. The values were constant within the limits of the precision of the assay, and the chromatographic peaks were clean with no shoulders or other indication of breakdown of the vitamins. In addition, four other single donor human serum pools, which were established in August, 1977; April, 1978; August, 1978; and April, 1979; and used as controls for the original colorimetric assay and later for the HPLC assay, were stable. Vitamin A values were constant during the year or more of use in the colorimetric assay and during the use of approximately one year in the HPLC assay. Vitamins A and E in serum were found to be stable to freezing and thawing (seventeen freezing and thawing cycles over a period of five weeks).

DISCUSSION

The measurement of serum vitamin A and vitamin E is a very appropriate application of HPLC. The procedure is fast and reliable with a minimum of downtime in our experience. As long as a guard column is used, the HPLC columns, which are relatively expensive, last almost indefinitely; thus, the only major continuing material expense is the eluting solvent, which amounts to about 12 ml of methanol per assay. The small serum sample requirement of 100 μl is especially convenient when infants' serum is involved. Interferences, to which colorimetric assays are often subject, are not a problem with HPLC. The precision with a coefficient of variation of between 3 and 4% for vitamin A is superior to the precision of the trifluoroacetic acid colorimetric assay with a coefficient of variation of between 6 and 7%, observed in this laboratory. Our stability studies suggest that it is unlikely that any error is introduced into the measurements because of degradation of the vitamins during storage of the serum. The simultaneous measurement of the two vitamins in the HPLC assay will make vitamin E measurement, which has not been done extensively in health surveys very convenient to do and might help to elucidate in humans the significance of the sparing effect of vitamin E on vitamin A observed in animals [11].

We use a single internal standard, retinyl acetate, while Bieri et al. [6] use both retinyl acetate and tocopheryl acetate as internal standards. Although it would seem to be desirable to have a structurally related internal standard for each measured metabolite, in practice, we have found that results equal in precision and accuracy are obtained with either one or two internal standards. Eliminating tocopheryl acetate as an internal standard shortens the chromatographic run time by 20% and also reduces by one the number of peaks to be measured.

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