CHROMBIO. 3151

SENSITIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF PLASMA VITAMIN E AND VITAMIN A USING AMPEROMETRIC AND ULTRAVIOLET DETECTION

MAY-LYNN HUANG, GILBERT J. BURCKART and RAMAN VENKATARAMANAN*

Clinical Pharmacokinetics Laboratory, School of Pharmacy, University of Pittsburgh, Pittsburgh, PA 15261 (U.S.A.)

SUMMARY

A highly sensitive high-performance liquid chromatographic assay using amperometric detection has been developed for measuring α-tocopherol in plasma. Other minor components of vitamin E (β-, γ- and δ-tocopherols) can also be detected by this method. All-*trans*-retinol is simultaneously monitored by UV detection at 313 nm. This assay requires only 50 μl of plasma for quantification of both vitamin E and A. The minimum detectable quantities for both α-tocopherol and retinol are 1.0 ng with corresponding signal-to-noise ratios of 4.0 and 3.5. The total chromatographic analysis time for each sample is less than 15 min. This assay has been used to determine the concentrations of vitamin A and E in plasma obtained from patients with liver disease, liver transplant patients and normal healthy volunteers.

INTRODUCTION

Vitamins are essential for normal growth and development of the human body and vitamin deficiency often leads to clinical abnormalities. Deficiency in certain vitamins can occur secondary to certain disease states. Malabsorption of vitamin A and E occurs in cystic fibrosis and in cholestatic liver disease [1, 2]. This malabsorption is due to impaired bile production and hence the lack of bile to facilitate the absorption of these fat soluble vitamins [3].

Measurement of plasma vitamin A and E provides an indication of the proper absorption of these two compounds. In the past, several analytical methods have been reported for the separate estimation of vitamin A and E in feed and in biological fluids [4—8]. However, very few methods have been published for the simultaneous estimation of these compounds in biological fluids. These

include fluorometric [9] and high-performance liquid chromatographic (HPLC) [10—13] methods. The HPLC methods published are based on the ultraviolet (UV) absorbance of vitamin A and E. All the methods published so far require a minimum of 100 or 200 μl of plasma for quantification of these compounds [10—13]. Malabsorption and the neurological sequelae of vitamin E deficiency are more commonly observed in infants and children than in adults. Since an adequate amount of plasma is difficult to obtain in the pediatric population, a sensitive assay that requires only small volumes of blood ($< 100 \mu$l) and that can simultaneously quantify both vitamin A and E is necessary.

We report a sensitive HPLC method for simultaneous measurement of vitamin A and E in plasma using a combination of amperometric and UV detection. The unique features of this assay are that it requires only 50 μl of plasma and that it uses essentially non-aqueous amperometric detection.

EXPERIMENTAL

Chemicals and reagents

All-*trans*-retinol and *dl*-α-tocopherol were purchased from Supelco (Bellefonte, PA, U.S.A.). *dl*-Tocol, *d*-β-, γ- and δ-tocopherols were obtained from Eisai (Tokyo, Japan).

HPLC-grade ethanol, *n*-hexane and sodium perchlorate were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). HPLC-grade methanol was obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.).

Laboratory precautions

All samples and analytical standards were protected from light by storage in aluminum foil-wrapped containers. Analysis of plasma samples was performed with minimum exposure of the samples to any light.

Chromatography

The HPLC system consisted sequentially of a Waters M-45 pump (Waters Assoc., Milford, MA, U.S.A.), a Waters U6K injector, a 5-cm guard column (40 μm, Supelco, Bellefonte, PA, U.S.A.), a 15 cm $\times$ 4.6 mm I.D. LC-18 analytical column (5 μm, Supelco), a Waters 440 UV absorbance detector with a 313-nm filter and an amperometric detector (BAS, West Lafayette, IN, U.S.A.) with a glassy carbon electrode and an Ag/AgCl (in 3 M sodium chloride) reference electrode. The working potential was set at +0.6 V vs. Ag/AgCl. The UV and electrochemical responses were recorded on a Fisher dual-pen recorder. A Hewlett-Packard 3390A integrator was used to integrate the electrochemical response.

Both the guard column and the analytical column were maintained at 31°C. The mobile phase was composed of 94% methanol in 0.05 M sodium perchlorate. Prior to use the mobile phase was filtered and degassed. The flow-rate was 1.5 ml/min.

The concentrations of α-tocopherol and retinol in each sample were determined using the following relationship: concentration of α-tocopherol or retinol = (peak height of α-tocopherol or retinol/peak height of tocol) $\times$ (1/slope of the standard curve for α-tocopherol or retinol).

Hydrodynamic voltammograms

Studies were carried out to determine the optimal potential setting required to monitor tocol, α-tocopherol and retinol. The potential between the working electrode and the surrounding mobile phase was varied from +0.10 to +0.95 V (vs. Ag/AgCl). At each potential setting, a fixed amount of tocol (21 ng), α-tocopherol (13 ng) or retinol (20 ng) was injected onto the column and the peak current generated was recorded. An optimal operating potential for the assay was selected on the basis of the results obtained.

Sample preparation

Blood samples were collected from normal healthy volunteers and patients using heparinized vacutainers. The plasma was immediately separated to minimize or prevent any hemolysis. Sufficient ethanol was added to all the plasma samples to obtain a final concentration of 2% (v/v). A 50-μl sample of plasma was then transferred to a 15-ml PTFE-capped centrifuge tube containing 50 μl of a methanolic solution of tocol (0.408 μg/ml). The mixture was vortexed for 10 s. The samples were extracted with hexane (4 ml) for 15 min at high speed in an Eberbach mechanical shaker. Following centrifugation at 1200 g for 5 min, the hexane layer was transferred to a round-bottom tube and evaporated under a stream of nitrogen in a water bath maintained at 30°C. The residue was dissolved in 100 μl methanol and 30 μl were injected onto the column.

Recovery study

A series of plasma standards were spiked with a known quantity of a methanolic solution of α-tocopherol and retinol to yield a final concentration in the range 0.1–21 μg/ml. These samples were extracted as previously described but without the use of tocol solution. After extraction, 2 ml of the hexane layer were transferred to a tube containing 50 μl of tocol solution (0.408 μg/ml). The mixture was evaporated and reconstituted with 100 μl methanol. A parallel series of methanolic solutions of retinol and α-tocopherol standards along with tocol were prepared and injected onto the column without any extraction. The percentage recovery after extraction was calculated as the ratio of the slope of the plasma standard curve to the slope of standard curve obtained by direct injection of the compounds without any extraction.

RESULTS

Fig. 1 shows the hydrodynamic voltammograms of α-tocopherol, tocol and retinol. The plateau current was reached at about +0.4 V and +0.55 V for α-tocopherol and tocol, respectively. Retinol initiates its electrochemical response at a potential of greater than +0.55 V and did not reach a steady-state voltammetric response within the potential applied in this study. Under the study conditions, retinol demonstrates a greater response in UV (313 nm) than in amperometry. We therefore monitored retinol at 313 nm, a wavelength which is specific to retinoids. A potential of +0.6 V was chosen for amperometric detection to minimize the background currents while
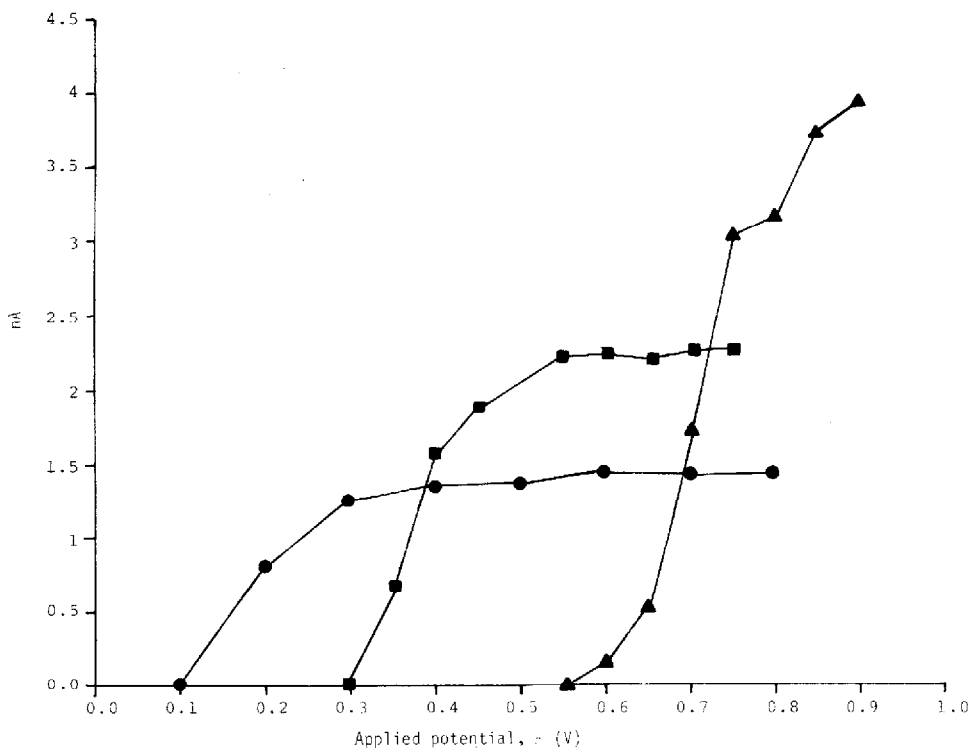
Fig. 1. Hydrodynamic voltammograms for α-tocopherol (13 ng) (●), tocol (21 ng) (■) and retinol (20 ng) (▲).

maintaining the plateau regions of both the voltammograms for tocol and α tocopherol.

Fig. 2 shows typical chromatograms of a plasma extract from a normal healthy volunteer. α-Tocopherol was separated from the other tocopherols. However, β- and γ-tocopherols were found to co-elute under the chromatographic conditions used. Retinol was the first compound of interest to be eluted and was well separated from the solvent front. The retention times for all-*trans*-retinol, tocol, δ-tocopherol, β- and γ-tocopherol and α-tocopherol were 3.0, 7.1, 9.0, 11.1, and 13.4 min, respectively. The minimum detectable quantity (on-column injection) for both α-tocopherol and retinol is 1.0 ng with corresponding signal-to-noise ratios of 4.0 and 3.5.

Linear calibration curves were obtained with characteristics and concentration ranges shown in Table I. The positive intercepts represent the endogenous vitamin E and A. This intercept varies with the origin of the blank plasma used. When calculating the unknown concentration, the peak-height ratio of the unknown was divided by the slope of the calibration curve. The within-day and the inter-day coefficients of variation were 7.1% ($n = 7$) and 12.1% ($n = 4$) for retinol and 4.8% ($n = 7$) and 0.89% ($n = 4$) for α-tocopherol. The mean recoveries ($n = 3$ at each concentration) were 78% for α-tocopherol (0.1—20 μg/ml), 98% for retinol (0.1—20.6 μg/ml) and 83% for tocol at 20.4 μg/ml.

The method developed was used to assay α-tocopherol and retinol concentrations in plasma samples obtained from normal healthy volunteers, patients
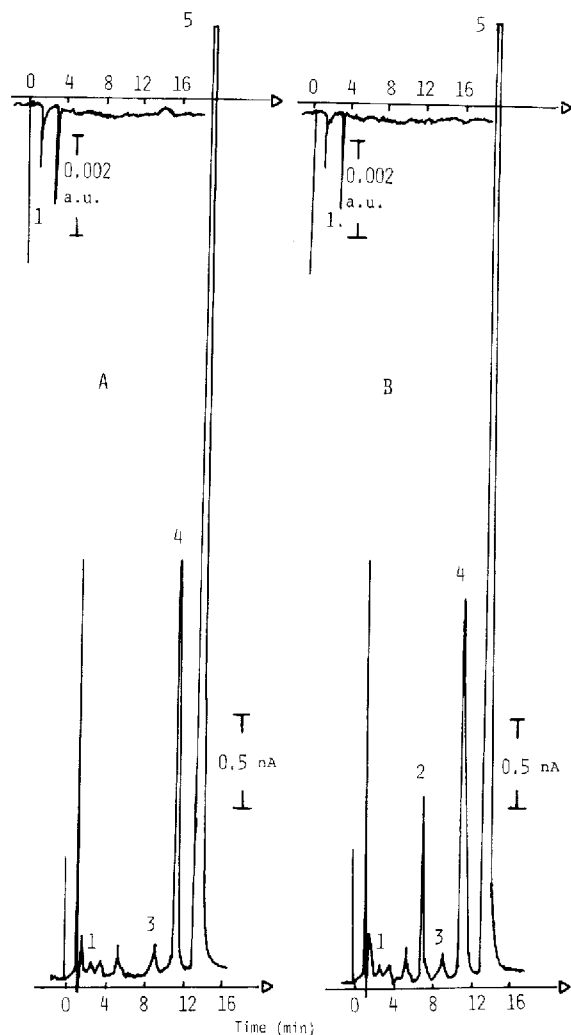
Fig. 2. Chromatograms of extracts from healthy human plasma (A) and human plasma spiked with 50 μl of tocol (0.41 μg/ml) as internal standard (B). Top chromatograms are UV absorptions at 313 nm. Bottom chromatograms are electrochemical responses at +0.6 V vs. Ag/AgCl. Peaks: 1 = retinol; 2 = tocol; 3 = δ-tocopherol; 4 = β- and γ-tocopherol; 5 = α-tocopherol.

TABLE I

CHARACTERISTICS OF PLASMA STANDARD CURVES

Compound	Regression line*	Correlation coefficient (r)	Concentration range (μg/ml)
All-*trans*-retinol	$y = 1.33x + 0.76$	0.999	0.10—20.8
α-Tocopherol	$y = 0.896x + 11.81$	0.993	0.10—20.6

*Peak-height ratio (y) versus spiked plasma concentration (x).

TABLE II

RETINOL AND α-TOCOPHEROL CONCENTRATIONS IN HUMANS

Subject No.	Concentration (μg/ml)					
	Normal subjects		Liver disease subjects		Liver transplant subjects	
	Retinol	α-Tocopherol	Retinol	α-Tocopherol	Retinol	α-Tocopherol
1	0.75	8.6	0.77	—	2.07	7.6
2	0.91	7.4	0.12	3.3	1.45	11.3
3	0.42	7.7	0.30	5.4	0.90	8.4
4	0.76	14.1	—	1.7	1.03	8.6
5	0.57	13.2	0.36	4.2	1.48	7.1
6	0.90	10.5	0.88	12.5	1.42	8.4
Mean ± S.D.	0.72 ± 0.19	10.2 ± 2.9	0.46 ± 0.34	5.4 ± 4.2	1.39 ± 0.41	8.6 ± 1.5

with liver disease and liver transplant patients. The results are shown in Table II.

DISCUSSION

Amperometric detection in combination with reversed-phase HPLC has become popular in clinical drug analysis owing to its high sensitivity. Most applications to date deal with compounds with low to medium lipophilicity. The mobile phase used in amperometric detection is predominantly aqueous. The use of organic mobile phases is rare [14]. The major reasons for this include the poor solubility of most electrolytes which are used for conductivity in organic solvents and the incompatibility of organic solvents with some commonly used working electrodes. We have demonstrated in this study that with the use of a glassy carbon electrode and the proper choice of an electrolyte, amperometric detection coupled with highly non-aqueous reversed-phase HPLC is possible for lipid soluble vitamins.

The low oxidation potential of the tocopherols yields itself to detection by amperometric methods. The amperometric detection of α-tocopherol is more sensitive than detection based on UV absorbance. A direct on-column injection of 10 ng of α-tocopherol produces no detectable peak with UV detection at 280 nm (0.001 a.u.f.s.) while a peak current of 1.1 nA is generated at an applied potential of +0.6 V (vs. Ag/AgCl). The estimated minimum detectable concentration of α-tocopherol, using 50 μl of plasma, is 0.85 ng/ml. In the present assay only a 30-μl aliquot of the 100 μl of the final reconstituted sample is injected onto the column. The minimum detectable concentration can be further improved by injecting a larger volume of the reconstituted solution. The linear range of the standard curve in our assay is nearly 200-fold as compared to a range of 5- to 10-fold in previous assays.

Two assays for measurement of plasma α-tocopherol using electrochemical detection have recently been published [7, 15]. The mobile phase used in these publications consisted mainly of methanol with either sodium perchlorate or acetate as the electrolyte. The detector was operated at +0.7 V or +1.0 V (vs. Ag/AgCl), respectively. In our assay we used a lower oxidation potential (+0.6 V vs. Ag/AgCl). Under our conditions we were able to detect compounds

at a more sensitive scale (2 nA or 5 nA full scale) with less than 1 nA background current.

Our assay also differs from previously published assays in the choice of internal standard used. Both the published assays use δ-tocopherol as the internal standard. Since large quantities of δ-tocopherol occur in a number of vitamin E supplements, these assays cannot be used in patients on vitamin E therapy. Tocol, on the other hand, is an analogue of vitamin E and has a relatively low oxidation potential (Fig. 1). It is not present in food or diet supplements and has chemical properties similar to α-tocopherol. These factors make it an ideal internal standard for vitamin E assay.

Compared to α-tocopherol, retinol is not as readily oxidized (Fig. 1). However by monitoring retinol at 313 nm, the sensitivity can be improved by a factor of three as compared to previously published methods [11, 12]. The estimated minimum detectable concentration of retinol using 50 μl plasma is 0.68 ng/ml. The linear range of the standard curve is nearly 200-fold.

Due to the increased sensitivity of detection of both α-tocopherol and retinol, these compounds can be quantified in a small volume of plasma (50 μl). The total chromatographic analysis time of a sample is 15 min. When vitamin E alone is analyzed, the composition of methanol in the mobile phase can be increased to shorten the analysis time to less than 8 min.

In preliminary studies, we analyzed vitamin E and A amperometrically with a phenyl column and used a 60% organic mobile phase [16]. This present method provides a better separation of retinol from the solvent front, an improved resolution of the tocopherols, increased sensitivities for both compounds studied and shorter analysis time compared to our earlier report.

Our studies also show no difference in the plasma concentration of α-tocopherol when the blood samples from a healthy volunteer were centrifuged at different temperatures [10.52 ± 0.22 μg/ml (25°C) versus 10.56 ± 0.63 μg/ml (37°C)]. Unlike cyclosporine, the partition of α-tocopherol into erythrocytes and uptake by lipoproteins is probably not sensitive to temperature changes [17].

We have used the method to analyze samples from normal, liver disease and post-liver transplant patients. In several liver disease patients, the vitamin E and A concentrations are lower than in normal subjects. In liver transplant patients, normal plasma concentrations of both the nutrients were observed. This reflects the ability of the transplanted liver to produce the required endogenous substances needed to facilitate the absorption of both retinol and α-tocopherol. The presently described assay is being used in a bioavailability study of vitamin A and E in patients pre- and post-liver transplantation.

REFERENCES

1   R.J. Sokol, M.K. Farrell, J.E. Heubi, R.C. Tsang and W.F. Balistreri, J. Pediatr., 103 (1983) 712.
2   P.M. Farrell, J.G. Bieri, J.F. Fratantoni, R.E. Wood and P.A. di Sant'Agnese, J. Clin. Inv., 60 (1977) 233.
3   F. Weber, Int. Z. Vitam. Ernahrungsforsch., 25 (1983) 55.
4   H. Cohen and M. Lapointe, J. Agric. Food Chem., 26 (1978) 1210.
5   C.H. McMurray and W.J. Blanchflower, J. Chromatogr., 178 (1979) 525.

6   A.P. De Leenheer, V.O. De Bevere and A.E. Claeys, Clin. Chem., 25 (1979) 425.
7   M. Vandewoude, M. Claeys and I. De Leeuw, J. Chromatogr., 311 (1984) 176.
8   M.G.M. De Ruyter and A.P. De Leenheer, Clin. Chem., 22 (1976) 1593.
9   J.N. Thompson, P. Erdody and W.B. Maxwell, Biochem. Med., 8 (1973) 403.
10 J.G. Bieri, T.J. Tolliver and G.L. Catignani, Am. J. Clin. Nutr., 32 (1979) 2143.
11 A.P. De Leenheer, V.O.R.C. De Bevere, M.G.M. De Ruyter and A.E. Claeys, J. Chromatogr., 162 (1979) 408.
12 W.J. Driskell, J.W. Neese, C.C. Bryant and M.M. Bashor, J. Chromatogr., 231 (1982) 439.
13 A.T. Rhys Williams, J. Chromatogr., 341 (1985) 198.
14 W.L. Caudill, M.V. Novotný and R.M. Wightman, J. Chromatogr., 261 (1983) 415.
15 P.P. Chou, P.K. Jaynes and J.L. Bailey, Clin. Chem., 31 (1985) 880.
16 M.L. Huang, G.J. Burckart and R. Venkataramanan, Am. Pharm. Assoc. Acad. Pharm. Sci., 14 (1984) 217 (Abstract).
17 M. Lemaire and J.P. Tillement, J. Pharm. Pharmacol., 34 (1982) 715.