

Note

Clinical and Analytical Evaluation of the Simultaneous HPLC Assay of Retinol and α -Tocopherol

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Summary We describe a method for the simultaneous assay of retinol and α -tocopherol using normal-phase, high-performance liquid chromatography (HPLC). Our normal-phase HPLC method gave better resolution (R_s) of retinol ($R_s=1.58$) and α -tocopherol ($R_s=1.40$) when compared with the R_s values for α -tocopherol and retinol from literature. Also, the α -tocopherol concentrations obtained by our method agreed well with another normal-phase HPLC method that used fluorometric detection ($r=0.951$, $p<0.001$, $Sy.x=0.58$ mg/L). The concentrations of retinol in our method agreed well with those determined by a reversed-phase HPLC procedure, although the correlation ($r=0.646$, $p<0.001$, $Sy.x=62$ μ g/L) was not as good as the method proposed. Our procedure gave acceptable precision: the within-run CV was 7.7% for α -tocopherol and 5.9% for retinol. The between-day CV was 9.0% for α -tocopherol and 6.8% for retinol. The mean recoveries were 97% for α -tocopherol and 107% for retinol. Our assays were linear for α -tocopherol concentrations from 0.1 to 30 mg/L and for retinol concentrations from 20 to 2,000 μ g/L. In children ages 7 to 12 y, and in adolescents ages 14 to 16 y, the α -tocopherol and retinol concentrations in the blood were significantly lower than the concentrations in normal adults. Individuals over 70 y old also showed α -tocopherol and retinol values that were lower than those of normal adults between ages 30 and 40 y. In female university students, the inter-individual variation of α -tocopherol was reduced by dividing the α -tocopherol results by their total cholesterol or total lipid concentrations; however, this was not obtained for retinol. In cancer patients undergoing surgery, the ratio of retinol to retinol-binding protein (RBP) remained fairly constant, although the concentrations of both retinol and RBP decreased to about one-half the preoperative values after surgery. We conclude that our normal-phase HPLC method is a stable and reproducible method for α -tocopherol and retinol, and is an easy-to-use analytical tool.

Key Words high-performance liquid chromatography, inter-individual variation, reference intervals, retinol, α -tocopherol

Retinol, retinoic acid, and retinal are essential nutrients. Retinol is required for normal reproduction, growth, and vision. Retinoic acid is required for the transport of mannose units in the synthesis of certain glycoproteins, and it also regulates gene expression in antiviral, antitumor, and immunomodulatory processes (1). Retinal plays a primary role in maintaining normal vision. Dietary vitamin A is absorbed by the intestinal mucosa, and more than 90% of the absorbed vitamin accumulates in the liver. Retinol, released from hepatocytes, combines with plasma retinol-binding protein that in turn combines with circulating prealbumin in a

1:1 molar ratio (2). The serum concentrations of retinol remain fairly constant until liver stores are nearly exhausted (3). Serum retinol concentrations are usually decreased in patients with liver, pancreatic, or gastrointestinal diseases, hyperthyroidism, sterility, in teratogenesis, chronic infections, zinc deficiency, and cystic fibrosis (4).

Vitamin E consisting of the α -, β -, γ -, and δ -tocopherol isomers, affects several normal functions. It protects polyunsaturated fatty acids from auto-oxidation within lipid and phospholipid membranes, it inhibits the oxidation of low-density lipoproteins, and protects against oxidative injury. Vitamin E also plays an important role in the immune response, and reduces the propensity of platelets to adhere to the blood vessel wall (2, 5). Dietary vitamin E is absorbed from the small intestine and enters the bloodstream via the lymphatics

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Abbreviations: CV, coefficient of variation; HPLC, high-performance liquid chromatography; RBP, retinol-binding protein; TPN, total parenteral nutrition; UV, ultraviolet.

where the α -tocopherol combines with chylomicrons. The vitamin is stored in most tissues and cells such as adipose tissue, hepatocytes, erythrocytes, and platelets (6). α -Tocopherol also binds to the lipoproteins present in cellular membranes. A rapid transfer of α -tocopherol occurs between the erythrocyte membranes and plasma lipoproteins (2). Serum or plasma concentrations of α -tocopherol, the most active isomer biologically, are decreased in premature infants with abetalipoproteinemia, in most patients with steatorrhea, biliary atresia, cirrhosis, cystic fibrosis, chronic pancreatitis, pancreatic carcinoma, gluten enteropathy, regional enteritis, chronic cholestasis, and other disorders (4). The cellular concentrations of α -tocopherol are a better measure of vitamin E nutriture because cellular concentrations are more sensitive to vitamin E intake and are not dependent on circulating lipoproteins (4). Recent epidemiological studies found a decrease in risk of ischemic heart disease with the increased intake of vitamin E. Significant protection began at serum α -tocopherol concentrations of more than 13 mg/L (5). Obviously, accurate and precise assays for α -tocopherol are needed to determine the vitamin E status of a patient. The assay of α -tocopherol in serum or plasma is simpler to carry out than the test on whole blood.

On the basis of the above clinical needs, reversed-phase HPLC employing C_{18} columns was proposed elsewhere as a selected method for the simultaneous assay of retinol and tocopherol isomers (7). The reversed-phase method uses 0.1 mL serum or plasma. It is rapid, simple, and inexpensive; however, it gives incomplete separation of tocopherol isomers and retinol. With this method, a satisfactory resolution (R_s) was obtained for the α -tocopherol peak ($R_s=0.91-1.11$), but not for the retinol peak ($R_s=0.75-0.95$) (7-9). Also the published reversed-phase HPLC method exhibited a baseline drift during isocratic elution. Thus, we developed a normal-phase HPLC method that provides a complete separation of retinol and α -tocopherol.

The goal of the present study was to evaluate the applicability of our method to serum (or plasma) specimens from normal volunteers and patients before and after cancer surgery.

Materials and methods

Liquid chromatography.

Normal-phase HPLC with UV detector. We used a L-7100 pump, L-7400 UV-detector, L-7300 column oven, and D-7500 integrator, all from Hitachi, Ltd., Japan. We also used a Shim-pack FLC-SIL column, 50×4.6 mm inner diameter and $3\text{-}\mu\text{m}$ particles, all from Shimadzu (Kyoto, Japan). This is for our proposed simultaneous measurement method of retinol and α -tocopherol.

Normal-phase HPLC with fluorescence detector. We used the same equipment as above but with a 821-FP Intelligent spectrofluorometer as the detector (Japan Spectroscopic Co., Ltd.), and a C-R6A Chromatopac from Shimadzu. This HPLC method was used as the comparison assay for α -tocopherol and tocopherol iso-

mers.

Reversed-phase HPLC with UV detector. We performed the silver-ion-free reversed-phase HPLC method as described earlier (10) as the comparison assay for retinol. We used the same equipment as for the normal-phase system above but with a SPD-6AV UV-VIS spectrophotometric detector and a C_{18} reversed-phase column with a 150×4.6 mm inner diameter and $5\text{-}\mu\text{m}$ particles (STR ODS-II), all from Shimadzu. The published reversed-phase HPLC method (7) was not used by us because of the incomplete separation of retinol.

Reagents. Hexane, 2-propanol, ethanol, and 2-methyl-2-phytyl-6-hydroxychromane (DL-tocol: as the internal standard) were of analytical grade from Wako Pure Chemical Industries, Ltd., Japan. All-trans-retinol was purchased from Sigma Chemical Co., St. Louis, MO, USA. The α -, β -, γ -, and δ -isomers of tocopherol and 2,2,5,7,8-pentamethyl-6-hydroxychromane (PMC) were purchased from Eisai Co., Ltd., Japan. All reagents were the highest available quality and were used without further purification. Working standards of α -tocopherol and retinol were prepared in 95% ethanol. The concentration of each working standard was confirmed spectrophotometrically using 1 cm quartz spectrophotometric cells with an ethanol blank. Retinol has an absorptivity of 1,780 at 325 nm, and for α -tocopherol, this is 75.8 at 292 nm (7).

Procedure. In our normal-phase HPLC method for the simultaneous assay of retinol and α -tocopherol, transfer 0.2 mL of serum (or plasma) or working standards of retinol, 1,000 $\mu\text{g/L}$, and α -tocopherol, 10 mg/L, to a 15.4×100 -mm glass test tube. Add 1.0 mL of water, 2.0 mL of ethanol, and 5.0 mL of hexane. Mix gently and centrifuge at $2,000 \times g$ for 10 min. Transfer 4.0 mL of the hexane layer to another 15.4×100 -mm glass test tube, and evaporate the hexane under a stream of nitrogen at 40°C . Dissolve the residue in 0.1 mL of hexane, inject 18 μL of the solution into the HPLC. Set the HPLC to 30°C and the flow rate to 0.8 mL/min using a hexane as eluent: 2-propanol solution at a ratio of 99.1 : 0.9 by volume. Monitor the effluent at 295 nm. Integrate the peak areas and use the values from the working standards to calculate the concentrations of retinol and α -tocopherol in the unknowns. When using the HPLC with a fluorometric detector, set the excitation wavelength at 298 nm and the emission wavelength at 325 nm. Calculate the resolution (R_s) as follows:

$$R_s = 2 \times [(t_{R2} - t_{R1}) / (w_1 + w_2)]$$

The terms, t_{R1} and t_{R2} are the retention times for solute 1 and 2, respectively, measured at the position of the peak. The terms w_1 and w_2 are the peak width in units of time measured at the base for solutes 1 and 2, respectively. The width of the peaks at the base is obtained by extending the sides of the peak to the zero concentration point as is shown in Fig. 1. If the R_s value for two adjacent peaks is more than 1.25, the peaks are considered to be well resolved (11).

Other measurements. Total cholesterol, triglycerides,

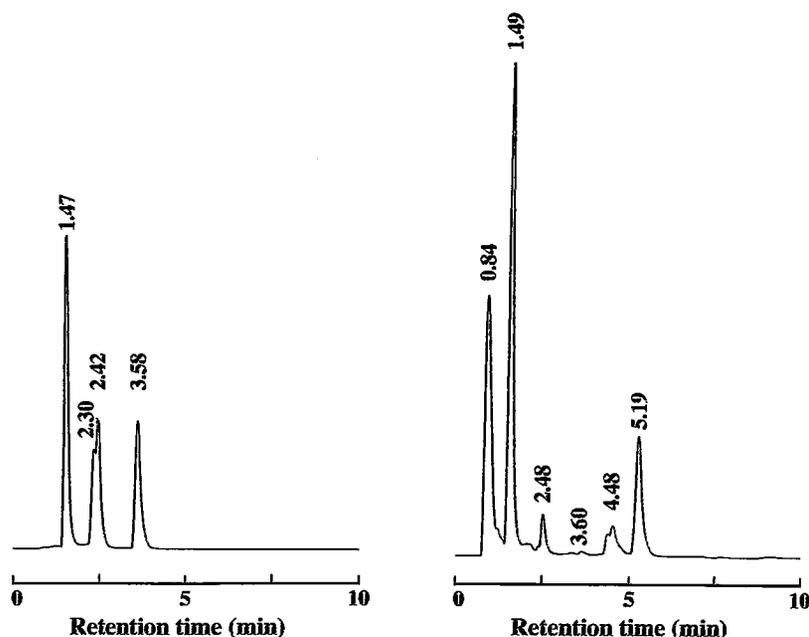


Fig. 1. Separation of the tocopherol isomers and retinol on normal-phase HPLC. Left: A chromatogram of a standard mixture containing (retention time) 10.0 mg/L α -tocopherol (1.5 min), 2.6 mg/L β -tocopherol (2.3 min), 2.8 mg/L γ -tocopherol (2.4 min), and 2.4 mg/L (3.6 min) δ -tocopherol. Right: A chromatogram of the tocopherol isomers and retinol in serum. Peaks at 5.2 min corresponded to retinol, 1.5 min to α -tocopherol, and 3.6 min to δ -tocopherol. β -tocopherol coeluted with γ -tocopherol at 2.5 min. Peak at 0.8 min contained retinyl palmitate and carotenes.

and phospholipids were measured in Olympus AU-5232 and AU-800 automated analyzers with in-vitro diagnostic kits for cholesterol from Daiya Shiyaku, Japan; triglycerides were determined by the method from Eiken Chemicals, Japan; and phospholipids with the Merck kit method (Merck, Germany). Retinol-binding protein (RBP) and prealbumin were determined by an immunonephelometric procedure (12). Our results on left-over survey serum for these lipids were all within the acceptable limits of the College of American Pathologists (CAP) and the Japan Medical Association surveys. The total lipid values were, calculated, with all the concentrations in mg/L, with the following equation (13):

$$\text{Total lipids in mg/L} = 1.5 \times \text{total cholesterol} \\ + \text{triglycerides} + \text{phospholipids}$$

Specimens. We collected blood specimens by vein puncture from 40 males, ages 7 to 90 y, and from 48 females, ages 10 to 83 y, who were all in good health and living in the Chubu District of Fukui Prefecture. Additional blood specimens were collected from 54 healthy women who were university students in the Tokyo area. The students took 2,460 IU of vitamin A and 5.8 mg of vitamin E on each of three days prior to vein puncture. We also collected blood from seven cancer patients before and 1, 7, 14, 21, and 28 d after surgery for colon (3 patients) and stomach cancer (4 patients). Written informed consent was obtained from all participants in the study, and the protocol was approved by the Protection of Human Subjects Committee of Toho University.

Results

Evaluation of analytical methods. Figure 1 shows the HPLC chromatogram of a standard mixture of tocopherol isomers. Our normal-phase HPLC method resolved α -, β -, γ -, and δ -tocopherol with retention times of 1.5, 2.3, 2.4, and 3.6 min, respectively. α -Tocopherol and δ -tocopherol in serum were completely resolved; β - and γ -tocopherol were coeluted as a single peak at 2.5 min. Retinol in serum was eluted at 5.2 min, and retinyl palmitate and carotenes coeluted at 0.8 min (See Fig. 1). As an example calculation for the resolution of α -tocopherol and retinol from their adjacent peaks, we calculated the R_s values by comparing both the t_R and w of α -tocopherol or retinol with those of their nearest peak; i.e., α -tocopherol at 1.49 min and a peak at 0.84 min; retinol at 5.19 min and a peak at 4.84 min. The R_s values were 1.58 for retinol and 1.40 for α -tocopherol. In our normal-phase HPLC method, DL-tocol and PMC cannot be used as internal standards because an unknown compound in the serum coeluted with DL-tocol at 4.3 min. The peak area of PMC was 1.5 times larger in the presence of serum as compared to the area with PMC alone.

We obtained a linear relationship between the peak area and concentration for the following concentration ranges: for α -tocopherol, 0.1–30 mg/L; γ -tocopherol, 0.3–30 mg/L, δ -tocopherol, 0.1–30 mg/L; and retinol, 20–2,000 μ g/L. When α - and γ -tocopherol were measured using fluorometric detection, a linear calibration range was found from 0.01 to 30 mg/L for α -tocopherol and 0.2 to 30 mg/L for γ -tocopherol.

The within-run precision of our method on pooled serum was as follows: mean (SD and CV%) for 10 repli-

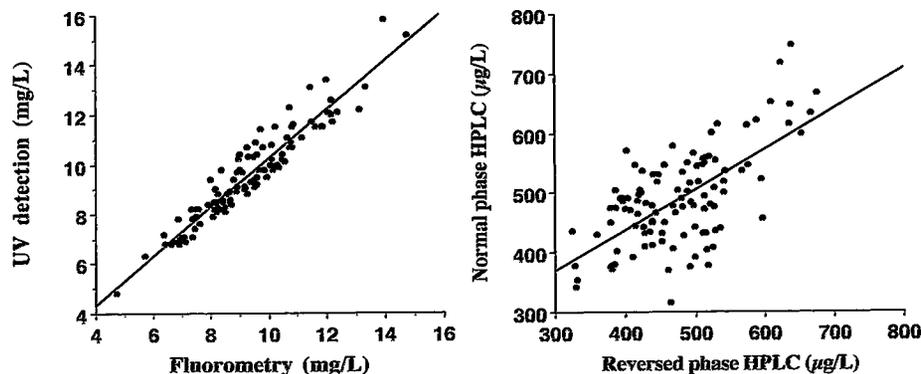


Fig. 2. Correlation of our normal-phase HPLC method with another HPLC method. Left: Comparison of UV and fluorometric detection of the effluent for the α -tocopherol in 107 plasma specimens, $r=0.951$. The regression equation is, $Y=0.98x+0.4$, $Sy.x=0.58$ mg/L. Right: Comparison of the normal-phase and reverse-phase HPLC methods for retinol in 107 plasma specimens, $r=0.646$. The regression equation is, $Y=0.68x+168$; $Sy.x=62$ μ g/L.

gives 15.64 mg/L, (1.21, 7.7) for α -tocopherol; 0.88 mg/L (0.06, 6.8) for γ -tocopherol; and 643 μ g/L (38, 5.9) for retinol. The fluorometric method gave comparable precision statistics: 15.22 mg/L (0.87, 5.7) for α -tocopherol and 2.42 mg/L (0.14, 5.8) for γ -tocopherol. Retinol is not fluorescent at the excitation wavelength of 298 nm. The mean value on the pool for γ -tocopherol obtained with fluorometric detection was significantly higher than that obtained with the UV detector ($p<0.001$).

The between-day precision for the same serum pool after storage in the dark at -20°C was 13.84 mg/L (1.24, 9.0) for α -tocopherol, 0.69 mg/L (0.05, 7.2) for γ -tocopherol, and 592 μ g/L (40, 6.8) for retinol. The same precision study with the fluorometric method gave 14.01 mg/L (1.03, 7.4) for α -tocopherol and 2.29 mg/L (0.25, 10.9) for γ -tocopherol.

Analytical recovery was estimated by analyzing a serum pool supplemented with six different concentrations of α -tocopherol, γ -tocopherol or retinol. Mean recoveries were acceptable for both α -tocopherol (by normal-phase HPLC, 97% with UV detector and 106% with fluorescence detector) and retinol (107% by normal-phase HPLC with UV detector); however, our recovery data suggested that γ -tocopherol was overestimated by the normal-phase HPLC method regardless of the type of detector used (146% and 125% with UV and fluorescence detectors, respectively). We believe our method for α -tocopherol and retinol; γ -tocopherol in serum appears to be overestimated.

Results for the α -tocopherol of patients and volunteers obtained by our HPLC method were compared using UV and fluorometric detection. We found a good correlation between the two detectors; $r=0.951$, $p<0.001$, $Sy.x=0.58$ mg/L (Fig. 2). Retinol in the same 107 plasma specimens obtained by our method gave results that were not significantly different from those by the reversed-phase HPLC procedure; $r=0.646$, $p<0.001$, $Sy.x=62$ μ g/L (See Fig. 2).

Reference ranges. We determined the serum concentrations of α -tocopherol and retinol in our 88 healthy volunteers who were on an ad lib diet. We found no gender differences for α -tocopherol (mean \pm SD). Males

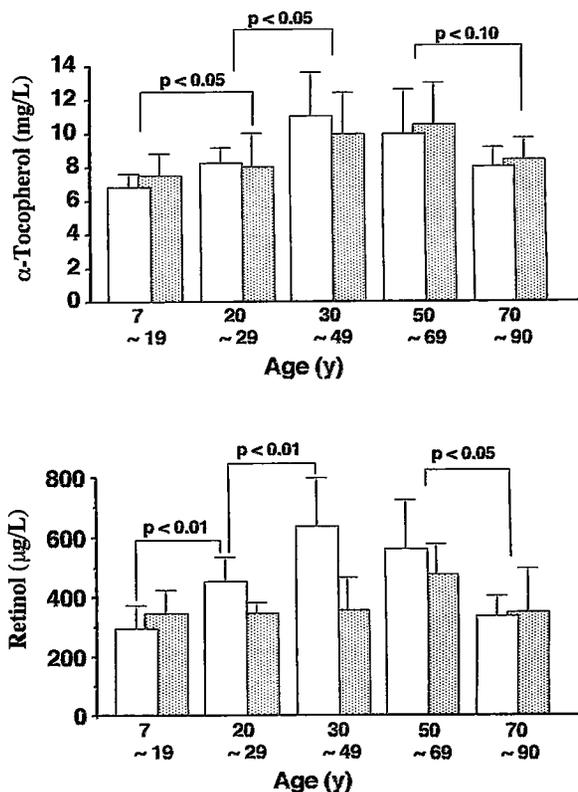


Fig. 3. Serum concentrations of α -tocopherol (upper) and retinol (lower) in individual age groups from 40 healthy males (white columns) and 48 healthy females (gray columns).

gave 9.2 ± 2.5 mg/L and females, 9.6 ± 2.4 mg/L, $p>0.05$. The males had significantly higher retinol concentrations, 503 ± 179 μ g/L, than the females, 383 ± 111 μ g/L, $p<0.001$. The serum concentrations of α -tocopherol and retinol in children and adolescents were significantly lower than those in adults, respectively (See Fig. 3). The retinol concentrations in older adults, ages 70 to 90 y, were significantly lower than those of younger adults ages 50 to 69 y ($p<0.05$). We also determined plasma concentrations of α -tocopherol and retinol in the 54 healthy young women who were university students and provided a dietary history. We

Table 1. Data from seven patients with colon or stomach cancer before and after surgery.

		Before surgery	Days after surgery				
			1	7	14	21	28
α -Tocopherol	mg/L	18.3 \pm 11.1	14.8 \pm 3.3	15.5 \pm 6.7	12.7 \pm 6.2	12.8 \pm 3.4	12.3 \pm 5.4
α -Tocopherol/Cholesterol	mg/g	7.0 \pm 2.7	9.4 \pm 4.2	8.3 \pm 4.5	6.4 \pm 2.6	7.4 \pm 1.2	6.8 \pm 1.7
Retinol	μ g/L	624 \pm 140	341 \pm 83***	330 \pm 80**	435 \pm 152*	434 \pm 160*	440 \pm 181*
Retinol-binding protein (RBP)	mg/L	44 \pm 11	25 \pm 11***	22 \pm 6***	32 \pm 12**	37 \pm 13	35 \pm 15*
Prealbumin	mg/L	314 \pm 47	227 \pm 26***	155 \pm 17***	233 \pm 50*	253 \pm 70	257 \pm 92
Retinol/RBP	molar ratio	1.05 \pm 0.16	1.09 \pm 0.36	1.13 \pm 0.25	1.04 \pm 0.27	0.92 \pm 0.28	1.02 \pm 0.35
Retinol/Prealbumin	molar ratio	0.42 \pm 0.06	0.32 \pm 0.06***	0.45 \pm 0.07	0.39 \pm 0.05	0.38 \pm 0.06	0.37 \pm 0.09

Statistical analysis was performed using the two-tailed paired *t*-test.

Differences were considered significant at $p < 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

found that their diets contained adequate amounts of vitamins A and E. The concentrations of α -tocopherol ranged from 6.8 to 15.8 mg/L (mean, SD of 10.1 mg/L, 1.9 mg/L) and retinol ranged from 288 to 602 μ g/L (424 μ g/L, 66 μ g/L). No significant difference ($p > 0.05$) was observed either in α -tocopherol or retinol concentrations between the 54 students and age- and gender-matched healthy volunteers. We estimated the reference intervals for the students using log-normal distribution curves. From these, we estimate their reference range to be 5.9–13.2 mg/L for α -tocopherol and 277–544 μ g/L for retinol. After dividing the students' α -tocopherol with their total cholesterol or total lipid concentrations, the reference interval was 4.33 to 7.42 mg/g total cholesterol and 1.42 to 2.42 mg/g total lipid, respectively.

Analysis of data from patients. Table 1 shows data from seven patients with colon or stomach cancer before and after surgery. All of the patients received total parenteral nutrition (TPN) that was not supplemented with vitamins A or E. These patients were allowed to take meals at seven days after surgery. There were no significant changes in α -tocopherol (nor in the ratios of α -tocopherol to total cholesterol concentration) when pre- and post-operative values were determined ($p > 0.05$). In these patients, the serum concentrations of retinol, RBP and prealbumin were significantly decreased especially at 1, 7 and 14 d after surgery as compared with their pre-operative values. At 14 d after surgery, the molar ratios of retinol to RBP were 1.1 or close to the values found pre- and immediately post-surgery. These ratios were not significantly changed ($p > 0.05$). The molar ratios of retinol to prealbumin were significantly decreased at 1 d post-surgery, but returned to the pre-operative concentrations on day 7.

Discussion

With our normal-phase HPLC method, we achieved complete separation of α -tocopherol and retinol in contrast to the earlier published work. Our method also showed good analytical performance with acceptable linearity, precision, recovery, and analytical specificity. These results were satisfactory as compared with the

previous HPLC procedure. We found a good correlation for α -tocopherol assayed by our HPLC method and the reversed-phase HPLC procedure. For retinol, the correlation of our method with the reversed-phase procedure was not as good. We deduced that the poor agreement in the assay of retinol by the two methods was probably owing to interfering substances (e.g., lipid-soluble compounds) and possibly the appearance of UV-absorbing polar substances such as retinoic acid or retinyl esters that coeluted with retinol (8). Regardless of these biases for the assay of retinol, we preferred the normal-phase HPLC method as a routine method for α -tocopherol and retinol because of the lower imprecision, ease of use, and elution from the column was completed in 6 min. Other HPLC procedures reported a 9 min elution time (7). The previous HPLC procedure used two internal standards (i.e., retinyl acetate and α -tocopheryl acetate) which increased chromatography run time.

With our normal-phase HPLC method, we observed that the serum concentrations of α -tocopherol and retinol in children and adolescents were significantly lower than in adults, a finding that has also been reported by others (4, 14). We also found that serum concentrations of α -tocopherol and retinol were decreased in those over 70 y old when compared with adults between ages 50 and 69 y for retinol and for α -tocopherol. The supposition is that older Japanese individuals consume less fat, and that the decreases found in blood are owing to the reduced α -tocopherol and retinol in their diet. We also found that retinol concentrations in males, ages 30 to 49 y, were significantly higher than females at the same ages. At ages of 20–29 and 50–69 y, the retinol concentrations were higher in males than females.

When plasma concentrations of α -tocopherol and retinol were determined in 54 female university students, the inter-individual variation (expressed as $100 \times \text{SD}/\text{mean}$) of α -tocopherol was 18.8%; the ratio decreased to 13.1 and 13.4% when divided by their total cholesterol or total lipid concentrations, respectively. Horwitt et al. (15), Farrell et al. (16) and Sokol et al. (17) reported that vitamin E deficiency should be evaluated only after correction for cholesterol or total

lipid concentrations. Vitamin E deficiency often existed with normal serum α -tocopherol concentrations. Jordan et al. (18) also suggested that retinol and α -tocopherol values required adjustment for the serum cholesterol or triglyceride concentrations. We corrected the plasma retinol and α -tocopherol concentrations by dividing by the total cholesterol, triglycerides, cholesterol plus triglycerides, or total lipid concentrations. We found that the CVs for α -tocopherol in plasma were 18.9 (unmodified) and 13.1% when divided by their cholesterol, 51.2% when divided by triglycerides, 15.7% when divided by the sum of the cholesterol and triglycerides, and 13.2% when divided by their total lipid concentrations. We concluded that dividing by total cholesterol or total lipid is appropriate and needed for the true α -tocopherol concentrations. We did not make similar findings for retinol concentrations; the ratios were not smaller when compared with the original values. In some cases, the ratios were larger after division by any of the above lipids or combinations of them.

We wondered if the plasma retinol concentrations were altered with changes in plasma RBP concentrations. In our cancer patients after surgery, both retinol and RBP concentrations were significantly decreased after surgery when compared with their preoperative values. The α -tocopherol concentrations did not change after surgery. All patients received TPN which did not contain vitamin A nor vitamin E. When the molar ratio of retinol to RBP was calculated, the ratio was quite constant at around 1.1 in the post-operative days. These observations indicated that plasma retinol values should be adjusted for plasma RBP concentrations. Muto (19) reported that one mole of plasma RBP combines with one mole of retinol. Our findings are largely in agreement with this, although we believe the ratio does not exceed 1.0.

We conclude that the major advantages of our method are the simplicity of the procedure, the speed of elution of the peaks, and better accuracy than the routine method in wide use. These attributes lead us to recommend our method for clinical use and research into the biochemistry and nutritional aspects of α -tocopherol and retinol.

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