Determination of Vitamin E in Microsamples of Serum by Liquid Chromatography with Electrochemical Detection

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In this procedure for determination of vitamin E by "high-performance" liquid chromatography with electrochemical detection, 25-μL serum specimens are deproteinized with ethanol. Vitamin E (α-tocopherol), its derivatives (β- and γ-tocopherols), and the internal standard (δ-tocopherol) are extracted into heptane and the extract is evaporated and the residue reconstituted with methanol before injection into the chromatograph. Within- and between-run CVs for an α-tocopherol concentration of 13.6 mg/L were 5.1% (n = 28) and 6.0% (n = 5), respectively. The standard curve is linear to 100 mg/L; the minimum concentration detectable is 0.1 mg/L. Analytical recovery ranged from 99.8% to 104.8%. In 36 specimens collected from apparently healthy subjects who were not taking vitamin supplements, α-tocopherol as determined by this method ranged from 4.3 to 9.7 mg/L, from 1.8 to 3.9 mg/L for β- and γ-tocopherols. Results by this method (y) and an HPLC-ultraviolet method (x) correlate reasonably (r = 0.81): y = 0.88x - 0.55 mg/L (n = 45). This procedure is adaptable to automated analysis, and the small sample requirement facilitates its applicability to neonates.

Additional Keyphrases: tocopherols · newborns · pediatric chemistry · reference interval

Deficiency of vitamin E (α-tocopherol) may cause various neuropsychological and myopathological abnormalities in children (1–4). Recently, neuromuscular disease in children has been associated with chronic vitamin E deficiency (5, 6). One probable cause of vitamin E deficiency is intestinal malabsorption, resulting from bowel disease, pancreatic disease, or chronic cholestasis.

Methods for determining α-tocopherol include ultraviolet spectrophotometry (7–9), gas–liquid chromatography (10, 11), and, more recently, "high-performance" liquid chromatography (HPLC) with ultraviolet spectrophotometric detection (12–18).

We wanted to develop a method that would permit measurement of vitamin E in relatively small samples. Electrochemical detection is one of the most sensitive HPLC detection methods, but the commonly used internal standard in HPLC spectrophotometric measurements, α-tocopherol acetate, is electrochemically inactive (19). Therefore, in our method δ-tocopherol is the internal standard.

Materials and Methods

Instrumentation. We used an HPLC pump (Model 8800; DuPont Instruments, Wilmington, DE 19888) equipped with an electrochemical detector (Model 4A; Bioanalytical Systems, West Lafayette, IN 47906) and an automatic sample injector (Model 710; Waters Associates, Milford, MA 01757). The 150 × 4.6 mm HPLC column (cat. no. 125-0081; Bio-Rad Laboratories, Richmond, CA 94804) was packed with 5-μm Bio-Sil ODS-SS. A guard column (cat. no. 125-0131) and a refill cartridge (cat. no. 125-0124) packed with a Bio-Sil ODS-SS (all from Bio-Rad Laboratories) was used to protect the analytical column. For comparison, we also used an ultraviolet detector (Model SF 770; Schoeffel Instrument Corp., Westwood, NJ 07675), to measure the absorbance of the HPLC effluent at 290 nm, according to the method of Driskell et al. (17).

Reagents. We used only distilled, de-ionized water. We purchased racemic α-, β-, and γ-tocopherols from Supelco, Inc., Bellefonte, PA 16823; δ-tocopherol was a generous gift of Hoffmann–La Roche, Nutley, NJ 07170. Absolute ethanol...
Sample preparation. Place 25 μL of specimen or control, 5 L of internal standard solution (δ-tocopherol, 100 μg per milliliter of ethanol), and 100 μL of ethanol in polypropylene vials (1.5-mL, with caps; cat. no. 50-407-5, Fisher Scientific Co.). Vortex-mix for 5 s and add 100 μL of heptane. Vortex-mix for 10 s, let the phases separate, and pipet 50 μL of the heptane (upper) layer directly into a glass insert (Waters Associates, cat. no. 73030) for the automatic sample-injection system. Evaporate the heptane fractions under a mild stream of air, then reconstitute each residue with 50 μL of methanol. Vortex-mix for 10 s, then place all the inserts in vials and cap the vials.

Chromatographic conditions. The flow rate was kept at 2 mL/min. Both the mobile phase and the HPLC column were kept at room temperature. The oxidizing potential was set at 1.0 V vs a Ag/AgCl reference electrode. The sensitivity range was 50 nA/V. The recorder range was 1.0 V for full-scale deflection; the chart speed was 0.25 cm/min. We injected 20-μL samples at 12-min intervals.

Results

To maximize the signal/noise ratio, we measured the change in the current strength of various tocopherols as a function of the oxidizing potential (Figure 1). On the basis of these results we set the oxidizing potential at 1.0 V. The pH of the sodium acetate solution in the mobile phase strongly affects the signal strength (Figure 2), a pH of 5.0 resulting in the maximum signal. We varied the percentage of aqueous solution in the mobile phase within the range of 0% to 15%. The mobile phase we selected satisfactorily separates α-tocopherol from β- and γ-tocopherol (Figure 3) and the last component is eluted from the column within a reasonable time, 11 min.

We calculate the concentrations of the α- or β- and γ-tocopherols by comparing their peak-height ratio with the internal standard in an unknown sample with that in a standard of known concentrations.

Fig. 2. Effect of the pH of the mobile phase on signal strength.

The pH of sodium acetate solution was adjusted with 6 mol/L HCl or NaOH solutions. Five volumes of this buffer solution plus 95 volumes of methanol was the final mobile phase. Symbols, concentrations, injection volume, and protocol as in Fig. 1.

Fig. 3. Typical chromatograms obtained for six different sera.

A, δ-tocopherol; C, α-tocopherol. Both β- and γ-tocopherol are co-eluted as one component (B). Sample 5 contains moderately increased α-tocopherol (45.6 mg/L); sample 6 has a very high α-tocopherol concentration (69.9 mg/L; top third of peak 6C has been cropped to save space).

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The within-run (n = 28 each) and between-run (n = 5 each) CV for \( \alpha \)-tocopherol at a concentration of 13.6 mg/L was 5.1% and 6.0%; for \( \beta \)- and \( \gamma \)-tocopherols at 2.4 mg/L, between-run CVs (n = 5 each) were 4.8% and 6.5%. The analytical recovery of 10, 20, and 40 ng of \( \alpha \)-tocopherol added to a sample was 104.8%, 99.8%, and 101.8%, respectively. To validate the procedure, we compared results obtained with it (y) with those by an HPLC–spectrophotometric method (x) in which tocopherol acetate is used as the internal standard (17). Linear regression of the data yielded a line with a slope of 0.88 and a y-intercept of \(-0.55 \text{ mg/L} (r = 0.81, n = 45).

Use of the present method to determine tocopherol concentrations in 36 specimens collected from 36 apparently healthy individuals (n = 5 each) who were not on vitamin supplements gave the following ranges: \( \alpha \)-tocopherol 4.3–9.7 mg/L, \( \beta \)- and \( \gamma \)-tocopherols 1.8–3.9 mg/L. Analysis of the same specimens by the comparison procedure (17) gave the following ranges: 4.2–14.0 and 0.3–4.9 mg/L, respectively. The minimum detection limits for the present method were 0.1 mg/L for all three derivatives.

**Discussion**

One function of vitamin E is to protect cell membranes against lipid peroxidation (20). More specific roles suggested for vitamin E in newborns include protection against: hyperbilirubinemia, retrolental fibroplasia, bronchopulmonary dysplasia, and intraventricular hemorrhage (19). Because concentrations of vitamin E in serum are generally lower in newborns than in adults (21), vitamin E supplementation for premature infants has become a very common practice. Typical colorimetric procedures for measuring vitamin E content require 1 mL of serum (7); recently developed HPLC–spectrophotometric procedures require 0.1 (17) to 1.0 (18) mL of serum. In the current procedure, a 25-\( \mu \)L sample (e.g., from heel punctures) is adequate, although the validity of using heel-puncture specimens has yet to be verified.

The use of \( \delta \)-tocopherol as internal standard has greatly improved both the precision and accuracy of our method. However, it obviates the use of this method for analysis of samples from individuals who are taking vitamin E supplements that may contain \( \delta \)-tocopherol. (\( \delta \)-Tocopherol makes up about 20% of the mixed tocopherols in soybean oil and 5% of those in wheat germ oil, and there is evidence of its presence in cottonseed and peanut oils (22).)

The reference ranges for tocopherols as determined by the two HPLC methods are slightly different. The higher values for \( \alpha \)-tocopherol as measured by ultraviolet detection suggests that that method may detect some interfering chromogens co-eluting with \( \alpha \)-tocopherol, and, indeed, we have noticed the co-elution of retinyl or retinoid esters with vitamin E derivatives under conditions similar to the ones used in this assay. These can give falsely high results if ultraviolet detection is used.

The extraction procedure has been simplified so that only one set of plastic vials is used, which eliminates pipetting error during a sample-transfer step. With this modification, 50 specimens can be processed in 2 h.

In summary: we find this procedure to be sensitive, specific, precise, and suited to routine use in clinical laboratories, especially in pediatric facilities, which need to process small samples.

**References**


