Simultaneous Liquid-Chromatographic Determination of Vitamin K₁ and Vitamin E in Serum

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We describe a high-performance liquid chromatographic procedure for the simultaneous measurement of vitamins K₁ and E in human serum. Delipidated human serum (free of vitamins K₁ and E) was used to make standard solutions of these vitamins, and cetyl naphthoate and α-tocopheryl acetate were the internal standards for vitamin K₁ and vitamin E, respectively. A simple, novel separation method utilizing liquid-liquid partition chromatography was used as a preparative "clean-up" procedure. Cetyl naphthoate and vitamin K₁ after post-column reduction) were detected by fluorescence, α-tocopheryl acetate and vitamin E by ultraviolet absorption. Sensitivity (detection limit) of the assay was 30 pg for vitamin K₁ and 5 ng for vitamin E per injection. The method is specific, precise, and more rapid than previously described procedures. Within- and between-assay CVs were 8.1% and 12.9%, respectively, for vitamin K₁; 3.5% and 6.0%, respectively, for vitamin E. Analytical recoveries of vitamins K₁ and E were 80% and 93%, respectively, from serum and from delipidated serum (standards). The average neonatal serum concentration of vitamin K₁ was 83 ng/L; 2.5 mg/L for vitamin E; for normolipidemic adults, the values were 343 ng/L and 1.9 mg/L, respectively, and for hyperlipidemic adults, 541 ng/L and 11.1 mg/L, respectively.

Additional Keyphrases: fat-soluble vitamins · lipids · cetyl naphthoate · α-tocopheryl acetate · fluorometry · newborns · hyperlipidemia · phyloquinone

The recent development of sensitive techniques of measuring vitamin K₁ in plasma has greatly assisted the understanding of factors influencing the availability, metabolism, and functions of vitamin K in animals and humans (1, 2). However, methods (3–9) currently available for determining physiological concentrations of vitamin K₁ in biological samples by "high-performance" liquid chromatography (HPLC) require several successive chromatographic steps to separate this vitamin completely from interfering lipids. Thus they are not practicable for use in routine analyses. For study of multiple plasma samples, such as population groups, a simplified technique is needed. Further, because vitamin K and vitamin E share a lipid transport system in plasma (10, 11) and are known to have a number of biological interactions (12–14), we have found it useful and convenient to monitor both vitamins in plasma simultaneously in various physiological and pathological states. Specifically, this has helped us detect clinical conditions involving changes in plasma phyloquinone concentrations, the result of altered metabolism of the carrier lipoproteins, rather than of vitamin K itself.

We report here a practical procedure for simultaneously measuring the fat-soluble vitamins K₁ and E in human serum by HPLC. A matrix of delipidated human serum (free of vitamins K₁ and E) is used in preparing standard solutions of these vitamins. A new internal standard (cetyl naphthoate) is used for the vitamin K₁ assay; α-tocopheryl acetate is used as the internal standard for the vitamin E assay. A simple, novel, preparative separation method, liquid-liquid partition chromatography, separates vitamins K₁ and E and their respective internal standards from other serum components. This clean-up procedure enables the quantification of vitamins K₁ and E in serum of hyperlipidemic patients. We have applied the method to the determination of vitamins K₁ and E in serum of newborns, healthy adults, and hyperlipidemic subjects.

Materials and Methods

Subjects

Blood samples were obtained from 20 normal adult subjects and 20 hyperlipidemic (serum cholesterol >5.5 mmol/L or serum triglyceride >1.75 mmol/L) subjects who had fasted for 12 h.

Blood samples (10 mL) were taken from the antecubital vein from fasting adults or from the umbilical cord vein from 70 neonates. The samples were protected from the light and were centrifuged at 4 °C. Serum samples (up to 3 mL) were extracted immediately or stored at −70 °C in disposable polystyrene tubes with polyethylene stoppers.

Serum pools were prepared from excess serum and were used for the studies of within-day coefficient of variation (CV), between-day CV studies, and recovery studies. They were also a source to prepare delipidated serum for making up standard solutions of the vitamins.

Instrumentation

HPLC was performed with a system incorporating a Model M-45 Solvent Delivery System, a Model 6000A Solvent Delivery System to supply the reducing agent, a Lambda-Max Model 481 LC spectrophotometer, a Guardpak Resolve C18 precolumn, a 5-nm-particle spherical C18 Resolve column (3.9 mm i.d. × 15 cm), a dual-channel Servogor 10 mV recorder (all from Waters Associates, Inc., Milford, MA 01757); a Rheodyne 7125 injection valve; an F-1000 Hitachi fluorescence spectrophotometer; and a stainless steel coil (0.8 mm i.d. × 1 m), which was used as a reactor.

Reagents

Methanol, isopropanol, and hexane (all HPLC purity) were purchased from Waters Associates, Brisbane, Australia. Ethanol (absolute) was purchased from CSL (Brisbane). Phyloquinone (all trans-vitamin K₁), α-tocopherol, α-tocopheryl acetate, and sodium borohydride were from Sigma Chemical Co., St. Louis, MO. γ-Tocopherol (biochemical grade) was from Fluka Chemie A. G., Buchs, Switzerland. 1-Naphthoic acid was from the Aldrich Chemical Co., Inc., Milwaukee, WI, and cetyl alcohol was from Merck, Darmstadt, F.R.G. Tritiated phyloquinone (all trans, specific activity 1.4 × 10¹⁵ dpm/g) was kindly donated by Hoffmann-La Roche, Baale, Switzerland. Distilled, de-ionized water was used throughout.
Standards. Pooled normal adult human serum was delipidated with a mixture of butanol-diisopropyl ether as previously described (15–17). This procedure removes lipids, including the fat-soluble vitamins (18), but does not precipitate proteins (including apolipoproteins) (15–18). The delipidated serum was extracted (see below, extraction of vitamins K₁ and E) and the sample extract was injected onto the column. Neither vitamin K₁ nor vitamin E was present in this extract.

A stock standard solution of vitamin K₁ was made up in ethanol (approximately 10 mg/L). The precise concentration was calculated by using a molar absorptivity coefficient of 19 900 L·mol⁻¹·cm⁻¹ at 248 nm for phylloquinone in ethanol. The concentration and purity of vitamin K₁ were routinely checked by multi-wavelength spectrometry with a Hitachi U-3200 UV-visible spectrophotometer (GEC, Brisbane, Australia) and by HPLC. Immediately before use, dilutions were made with isopropanol and added to delipidated serum to obtain aqueous standards (vitamin K₁ working standards, 0.0625 – 4.0 µg per liter of delipidated serum).

A stock standard solution of α-tocopherol (400 mg/L) was made up in isopropanol. The concentration and purity of vitamin E were routinely checked by multi-wavelength spectrometry and by HPLC. Appropriate portions of this solution were added to delipidated serum (which now also contained the vitamin K₁ standard) to obtain aqueous standards (α-tocopherol working standards, 0.625 – 40.0 mg per liter of delipidated serum). Working standards in delipidated serum were stored at -70 °C and were stable for at least nine months. All solutions containing vitamin K₁ were shielded from light.

Procedures

Synthesis of cetyl naphthoate. We esterified 24.2 g of cetyl alcohol with 17.2 g of 1-naphthoic acid in 200 mL of benzene, adding five drops of concentrated sulfuric acid as a catalyst. The mixture was refluxed for 3h, and the benzene was subsequently removed by distillation. The remaining solid material was dissolved in 200 mL of hexane and transferred to a separating funnel. We added 100 mL of de-ionized water to the hexane, inverted the mixture several times, then removed the water layer. After washing the hexane layer twice more with de-ionized water, we removed the hexane by evaporation in a vacuum chamber. This resulting crystalline residue (cetyl naphthoate, approximately 37 g), when chromatographed under the conditions described in this paper, gave a single peak by fluorometric detection, but was not detectable by ultraviolet absorption.

For the stock solution we used cetyl naphthoate (78 µg) and α-tocopheryl acetate (100 mg) dissolved in 1 L of isopropanol.

Extraction of vitamin K₁ and vitamin E. Serum specimens (usually 0.5–3 mL, depending on whether the specimen was from hyperlipidemic or newborn subjects) and working standards in delipidated serum were transferred to a borosilicate glass screw-cap (Teflon-lined) 16 × 125 mm culture tube; 7.8 ng of cetyl naphthoate and 10 µg of α-tocopheryl acetate in isopropanol (0.1 mL stock solution) were added as internal standards. Serum proteins were denatured by adding ethanol (2 mL per milliliter of serum), and the lipids were extracted into 5 mL of hexane. After mixing this solution in the dark for 15 min, we separated the two phases by centrifugation (3500 × g, 5 min). The upper (hexane) layer, containing vitamin K₁, vitamin E, the internal standards, and other lipids (not present in delipidated serum containing the standards), was transferred to a borosilicate glass screw-cap (Teflon-lined) 16 × 125 mm culture tube.

Clean-up of vitamin K₁, vitamin E, and internal standards. The hexane layer was “washed” by mixing with 5 mL of methanol:water (9:1 by vol) in the dark for 10 min. The two phases were separated by centrifugation (3500 × g, 5 min). The upper (hexane) layer was transferred to a disposable pointed borosilicate glass screw-cap (Teflon-lined) tube (17 × 125 mm), and the hexane was evaporated under a stream of nitrogen. The residue obtained after extraction was dissolved by addition of 120 µL of isopropanol; 100 µL of this was injected onto the column.

Analysis. For HPLC analyses of the vitamin K₁ and vitamin E, we injected 100 µL of the sample extract onto the column. The column was eluted isocratically with ethanol:water (92:8, by vol) at a flow rate of 0.5 mL/min at room temperature. The effluent from the column was mixed with the reducing agent (800 mg of sodium borohydride in 1 L of ethanol) and fed directly into a post-column reaction system at 55 °C in an SE Grant water bath (Grant Instruments, Barringdon, Cambridge, CB2 50Z, U.K.). This reduced the vitamin K₁, which we detected with the internal standard cetyl naphthoate by fluorescence spectrophotometry at an excitation wavelength of 320 nm, an emission wavelength of 430 nm, and a sensitivity setting range at ×20. The flow-rate of the reducing agent was 0.6 mL/min. The effluent from the fluorescence detector was fed directly into the LC spectrophotometer, where vitamin E and the internal standard α-tocopheryl acetate were detected at an absorbance wavelength of 292 nm and a sensitivity setting range at 0.1 A. Peak heights of the vitamins and their internal standards were recorded on a dual-channel chart recorder. Vitamin K₁ and vitamin E concentrations of the sample were measured by peak-height ratios with their corresponding internal standards and calculated from a calibration curve.

Recovery experiments. Known amounts of vitamin K₁ and α-tocopherol were added to delipidated serum samples and to serum samples that contained known amounts of endogenous vitamin K₁ and vitamin E. Total amounts were then measured and the analytical recovery was assessed. To calculate percentage recovery, the amount of endogenous vitamin was subtracted from the measured total amount, divided by the added amount, and multiplied by 100.

We also assessed extraction recoveries of [³H]phylloquinone (4800 counts/min) added directly to 1 mL of delipidated or nondelipidated sera in a scintillation vial. Control sera containing no radioactively labeled phylloquinone were also included. The serum samples were extracted and “cleaned up” as described above, the washed hexane layer was transferred into a scintillation vial and evaporated under a stream of nitrogen, and the residue was mixed with 10 mL of “Ready Safe” scintillant. We counted the radioactivity in the samples with a scintillation counter (Beckman), using external standardization and counting for 5 min at 35–50% efficiency, then calculated the recovery of the extracted radiolabeled phylloquinone.

Other methods. Serum cholesterol and triglyceride were measured by established procedures (19).

Treatment of data. For comparative statistical analysis of groups, we used Student’s t-test.
Precipitating serum proteins with ethanol facilitated the extractability of lipids by hexane. However, direct application of these extracted lipids to the HPLC system failed to separate vitamin K<sub>1</sub> from other lipids (Figure 1, left). Washing the hexane layer, which contains the extracted lipids, with a mixture of methanol/water (9/1, by vol) eliminated the interference of lipids and allowed good HPLC separation and quantification of vitamin K<sub>1</sub> (Figure 1, right) and vitamin E (Figure 2). Lipid extracts from the era of patients did not contain any substances that interfered with the detection of the internal standards (not shown). Cetyl naphthoate (retention time 7.0 min) was eluted just before vitamin K<sub>1</sub> (retention time 8.5 min) (Figure 1, right), whereas α-tocopheryl acetate (retention time 6.4 min) was eluted just after vitamin E (retention time 4.8 min) (Figure 2). Figure 1 (right) shows a chromatogram of a serum extract of vitamin K<sub>1</sub> from a normal subject (concentration 500 ng/L) with added cetyl naphthoate (7.8 µg/L). A large peak with a retention time of 26 min was present in the serum extract. The total HPLC time for quantifying vitamin K<sub>1</sub> and vitamin E was 30 min. Figure 2 illustrates a chromatogram of vitamin E (9.0 ng/L) and α-tocopheryl acetate (10 mg/L) in the same serum extract.

The reduction of vitamin K<sub>1</sub> by sodium borohydride was temperature dependent (Figure 3), there being a linear relationship in the peak-height ratio of vitamin K<sub>1</sub> to the internal standard cetyl naphthoate and temperature up to 0°C. We thus chose a reaction temperature of 55°C because this enabled appropriate reduction of vitamin K<sub>1</sub>. The delipidated serum used to make up the vitamin standards contained no traces of endogenous vitamin K<sub>1</sub> and vitamin E (Figure 4, right). It was feasible to get both good signal-to-noise ratio and selectivity towards the background signal from serum samples containing at least 30 g of vitamin K<sub>1</sub> on column. Increasing the length of the reaction coil to up to 2 m resulted in broadening of the peaks and did not improve the sensitivity of the method. In the absence of sodium borohydride, no peak was detectable at a retention time of 8.5 min.

Sensitivity of the assay for vitamin E was 5 ng on column. Standard curves for peak-height ratios for the pairs vitamin K<sub>1</sub>:cetyl naphthoate and α-tocopherol:α-tocopheryl acetate are shown in Figure 5. The regression equations of the standards made up in delipidated serum, calculated from peak-height ratios, were as follows: for vitamin K<sub>1</sub>, \( y = 2.46x - 0.19, r = 0.996, n = 7 \); for vitamin E, \( y = 3.97x + 0.18, r = 0.999, n = 7 \).

The results in Table 1 show the analytical recovery of vitamin K<sub>1</sub> and vitamin E from delipidated serum and nondelipidated serum. The recovery of the extraction of [3H]phylloquinone in delipidated and nondelipidated serum is also shown. Table 2 shows the results of our studies of precision, carried out with low and high serum vitamin K<sub>1</sub> and E concentrations. We also applied the proposed...
method to measure vitamins K\textsubscript{1} and E in sera from neonates, normal adults, and hyperlipidemic subjects (Table 3).

**Discussion**

Previously described HPLC assays required several successive tedious chromatographic steps to completely separate vitamin K\textsubscript{1} from interfering lipids and are not practicable for use in routine analysis (3–9). The proposed method specifically separates and quantifies vitamin K\textsubscript{1} accurately and rapidly. Sample preparation by deproteinization with ethanol and extraction of the vitamins in hexane, followed by a simple “washing” procedure with a mixture of methanol–water to eliminate interfering lipids simplifies and speeds the assay. The preparative clean-up step with the methanol–water wash before HPLC reduced the lipid load injected onto the LC column, so that we replaced the precolumn after ~100 injections, the analytical column after ~700 assays.

Under the described HPLC conditions, α-tocopherol is not separated from γ-tocopherol. This combined peak is represented here as vitamin E. α- and γ-tocopherol represent over 90% of vitamin E concentration in plasma, the ratio of the concentrations of α- to γ-tocopherol in serum being approximately 5 to 1 (20). The molar absorptivity coefficients (at wavelength 292 nm) for α-tocopherol in the mobile phase is 3058 L·mol\textsuperscript{-1}·cm\textsuperscript{-1}; for γ-tocopherol 3645. If required, complete separation of α-tocopherol from γ-tocopherol can be obtained by altering the composition of the mobile phase to ethanol–water (85/15, by vol). The retention times of α- and γ-tocopherol then become 10.8 and 8.6 min, respectively. Under these conditions, the retention times of cetyl naphthoate and vitamin K\textsubscript{1} are 17 and 25 min, respectively, and are quantifiable. However, the total HPLC running time then takes over 1 h, which is not feasible for routine analyses.

A newly described internal standard, cetyl naphthoate

<table>
<thead>
<tr>
<th>Table 2. Precision of Assay of Phylloquinone and Vitamin E in Serum</th>
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<tr>
<td><strong>Phylloquinone, ng/L</strong></td>
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<tr>
<td><strong>Within-run (n = 12)</strong></td>
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<td>Mean ± SD</td>
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<td>CV, %</td>
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<td><strong>Day-to-day (n = 8)</strong></td>
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<tr>
<td>Mean ± SD</td>
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<td>CV, %</td>
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**Table 1. Analytical Recovery of Phylloquinone and α-Tocopherol Added to Patients' Delipidated and Non-delipidated Sera (n = 4)**

<table>
<thead>
<tr>
<th></th>
<th>Phylloquinone, μg/L</th>
<th>α-Tocopherol, mg/L</th>
<th>[\textsuperscript{3}H]Phylloquinone, dpm</th>
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<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
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<tr>
<td>A, Standards added</td>
<td>1.00 ± 0.10</td>
<td>8.01 ± 0.24</td>
<td>14 479 ± 249.4</td>
</tr>
<tr>
<td>B, Delipidated serum</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>34.5 ± 4.1</td>
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<tr>
<td>C, Delipidated serum + standards</td>
<td>0.83 ± 0.10</td>
<td>7.47 ± 0.08</td>
<td>12 790 ± 207.3</td>
</tr>
<tr>
<td>% recovery [(C - B)/A] × 100</td>
<td>83 ± 6</td>
<td>93 ± 3</td>
<td>88 ± 5</td>
</tr>
<tr>
<td>E, Serum</td>
<td>0.19 ± 0.05</td>
<td>7.36 ± 0.32</td>
<td>39.4 ± 5.4</td>
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<tr>
<td>F, Serum + standards</td>
<td>0.98 ± 0.10</td>
<td>15.40 ± 1.17</td>
<td>12 251 ± 180.1</td>
</tr>
<tr>
<td>% recovery [(F - E)/A] × 100</td>
<td>80 ± 4</td>
<td>100 ± 12</td>
<td>84 ± 4</td>
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</table>
which is eluted just before vitamin K₄, contributes to the accuracy of the method. Internal standards used in the assay of vitamin K₁ by other investigators, e.g., menaquinone -6, are present in biological material (21). Cetyl naphthoate was synthesized chemically and we regard it as superior as an internal standard to endogenous compounds in biological materials.

Another important new approach is the solvent that we used for vitamin K₁ and a-tocopherol standards. Delipidated serum retains the endogenous carriers of lipid and fat-soluble vitamins, the apolipoproteins, but is devoid of the lipids and vitamins themselves (15–17). Thus the preparation of the standard curves and the assay of serum samples could follow an identical procedure, with the standard curve passing through the origin. Recovery studies showed that vitamin K₁ and vitamin E were extracted from delipidated serum and from patients' serum at equal rates. The absolute recovery of vitamin K₁ was ~80% and for vitamin E was ~93%.

Intra- and interassay repeatability studies have shown that the method is precise and reliable. Recovery studies show that the assay is accurate.

Values quoted for vitamin K₁ concentrations in plasma have tended to become lower as the sensitivity of the method used has increased (22). Our results (Table 3) closely agree with recent data for adults determined with electrochemical detection methods (0.2 – 0.7 μg/L, n = 45) (22). Conflicting data have been presented on vitamin K₁ concentrations in plasma from neonates. Values equivalent to adult concentrations have been quoted (6), but usually results have been much lower, ranging from 4 to 98 ng/L (22, 23). Our data (Table 3) yielded a value of 67 ± 46 ng/L (mean ± SD, n = 70), supporting the validity of the lower concentrations reported. Comparison of data from hyperlipidemic subjects—both vitamin K₁ and vitamin E—is rendered difficult by the heterogeneity of the tested population, but we saw a clearcut trend towards higher concentrations of both vitamins in these subjects, which is in accord with previous reports (10, 22).

We acknowledge the financial support of the National Health and Medical Research Council of Australia.

References