Liquid-Chromatographic Determination of Vitamin K₁ in Plasma, with Fluorometric Detection

Yacoob Haroon,¹ David S. Bacon,¹ and James A. Sadowski¹,²,³

This assay for phyloquinone (vitamin K₁) in plasma requires a single liquid-chromatographic step. Much smaller volumes of plasma (0.5–1.0 mL) are required than in previous assays. Before liquid chromatography, we purified crude lipid extracts by conventional chromatography on silica, then extracted the lipid fraction by dissolving it in an acidic mixture of hexane/acetonitrile (1/4 by vol) containing 70 mmol of zinc chloride per liter. The vitamin K₁ was selectively extracted into acetonitrile after being converted to vitamin K₁ hydroquinone by addition of zinc metal. This procedure removes >99% of contaminating lipids. We injected the lipid extract directly onto a reversed-phase column after re-converting the vitamin K₁ hydroquinone to vitamin K₁. Vitamin K₁ was quantified by comparison with the internal standard (dihydro-vitamin K₁) and detected fluorometrically after post-column “on-line” reduction to the hydroquinone with zinc metal. The lower limit of detection for vitamin K₁ in the final reversed-phase system was about 0.05 μg/L plasma; CVs for replicates were <10%. The mean concentration of vitamin K₁ in plasma from 22 healthy fasting adults was 0.55 (range 0.09–2.12) μg/L.

Additional Keyphrases: phyloquinone · reduction with zinc metal · chromatography, reversed-phase · reference interval

Vitamin K is required for the post-translational biosynthesis of γ-carboxyglutamic acid in several proteins (1). Several of these vitamin K-dependent proteins (Factors II, VII, IX, X, Protein C, and Protein S) play critical roles in hemostasis. Proteins containing γ-carboxyglutamic acid are also present in several other tissues (bone, kidney, lung, spleen, and testes) and in pathological specimens (ectopic calcified tissue and kidney stones). In humans, deficiency of vitamin K leads to decreased concentrations of circulating, active coagulation factors, which often results in bleeding. Vitamin K nutritional status has also been implicated in osteoporosis (2).

Reliable methods are available for determining other fat-soluble vitamins (i.e., vitamins A, D, and E, but not K) (3, 4) and their metabolites. These assays are suitable for routine analysis in a well-equipped clinical chemistry laboratory and do not require large sample volumes.

In contrast, the methods so far described for determining the low concentrations of vitamin K₁ in biological samples by liquid chromatography with ultraviolet detection (5–8) require several successive chromatographic steps to completely separate this vitamin from interfering lipids, and thus are not practicable for use in routine analysis. Electro-

chemical methods for detection of vitamin K have been of limited success (9–12).

Langenberg and Tjaden recently described a liquid-chromatographic assay for determination of vitamin K₁, in which fluorometric detection is used after post-column electrochemical reduction of the quinone (12). Vitamin K₁ has no native fluorescence, but the reduction product, vitamin K₁ hydroquinone, is highly fluorescent. However, this method relies on inefficient electrochemical cells to reduce the vitamin quantitatively.

Here we report a new method for assay of vitamin K₁ in biological samples that is faster and more sensitive than previous procedures. Fewer chromatographic steps are involved, and the post-column arrangement reduces 96% of the injected vitamin to its corresponding hydroquinone before fluorometric detection.

Materials and Methods

Chemicals. Vitamin K₁ (2-methyl-3-phytyl-1,4-naphthoquinone) was purchased from Sigma Chemical Co., St. Louis, MO. Vitamin K₁ with the 2', 3' double bond hydrogenated, "dihydro-K₁", was a gift from Drs. Brubacher and Weber (F. Hoffmann–La Roche and Co., CH-4002 Basel, Switzerland). Vitamin K₁-2,3-epoxide was synthesized from vitamin K₁ (13). High-purity acetic acid was obtained from Pierce Chemical Co., Rockford, IL, and high-purity 200-mesh zinc particles from Alfa Products, Danvers, MA.

Preparation of plasma samples. Plasma was sampled from 22 apparently healthy subjects, who had fasted overnight. Blood obtained by venipuncture was collected into tubes containing EDTA as an anticoagulant. After the plasma was separated, we pooled part of it from each subject and froze the rest for individual analyses. Specimens were collected in subdued light, and were kept in the dark during processing. All specimens were stored at −20 °C until analysis.

Extraction of vitamin K₁. After the specimens were thawed, we transferred 0.5–1.0 mL of plasma to a disposable borosilicate glass screw-cap culture tube (16 × 100 mm) and added 1.75 mg of dihydro-vitamin K₁ in ethanol (0.02 mL) as the internal standard. Plasma proteins were denatured by adding more ethanol (2.0 mL), and the lipids were extracted into 6 mL of hexane. After mixing and centrifugation (3500 × g, 5 min), two phases separated. The upper (hexane) layer, containing vitamin K₁, the internal standard, and other lipids, was transferred to a disposable borosilicate glass screw-cap 16 × 100 mm culture tube, and the hexane was evaporated at 30 °C in a vortex-type evaporator. All caps for the culture tubes were Teflon lined.

Silica chromatography. For preliminary purification of lipid extracts to remove polar lipids we used silica "Sep-Pak" columns (Waters Associates, Milford, MA 01757) as described previously (7). Briefly, the procedure is as follows. We dissolved the above-described lipid extracts in hexane

---

¹ Vitamin K Research Laboratory and ² Nutrition Evaluation Laboratory, USDA Human Nutrition Research Center on Aging at Tufts University, 711 Washington St., Boston, MA 02111.
³ Address correspondence to this author.
Received May 15, 1986; accepted July 25, 1986.

CLINICAL CHEMISTRY, Vol. 32, No. 10, 1986 1825
and applied this solution to the columns. Eluting with 8 mL of hexane removed the hydrocarbons. A "vitamin K₁-containing" fraction, including the internal standard and other lipids, was then eluted with 8 mL of a mixture consisting of 30 mL of ethyl ether per liter of hexane. All solvent was then evaporated from the samples in a vortex-type evaporator.

**Reductive extraction.** The lipid extract obtained after chromatography on silica was dissolved in 1.0 mL of hexane. We then added 4.0 mL of a "reductive extraction" solution (pH 2.1) containing 70 mmol of zinc chloride, 30 mL of acetic acid, and 970 mL of acetonitrile per liter. Next we added 5–10 mg of zinc metal, vortex-mixed the mixture for 2 min, centrifuged, and discarded the upper (hexane) layer, which contained the bulk of the contaminating lipids. We evaporated the lower (acetonitrile) layer containing the vitamin and dissolved the residue in 6.0 mL of hexane, then added 2.0 mL of water. After vortex-mixing and centrifuging, we placed the top (hexane) layer containing the vitamin K₁ into a disposable borosilicate screw-cap 16 × 100 mm culture tube and evaporated the solvent at 60 °C under a stream of air in a dry-block type of heater. We dissolved the residue in 250 μL of the mobile phase and injected 100 μL of this solution into the chromatograph. The data module and system controller automatically quantified vitamin K₁ by peak height ratios.

"High-performance" liquid chromatography. The liquid-chromatographic system consisted of a Model 510 reciprocating pump, a wise Model 710B automatic injector, a Model 721 system controller, and a Model 730 data module (all from Waters Chromatography, Division of Millipore, Milford, MA). Fluorescence (excitation at 248 nm, emission regulated with a 419-nm longpass cutoff filter) was measured with a "Spectroflow 980" programmable fluorescence detector (Kratos Analytical, Ramsey, NJ) set at 0.01 μA full scale. The 250 mm × 4.6 mm (i.d.) chromatographic column was packed with a 5-μm Hypersil-ODS (Shandon Southern Instruments, Inc., Sewickly, PA). The mobile phase contained 200 mL of dichloromethane and 800 mL of methanol per liter, to each liter of which we added 5 mL of a solution containing, per liter, 2.0 mol of zinc chloride, 1.0 mol of sodium acetate, and 1.0 mol of acetic acid. We used a flow rate of 1.0 mL/min.

**Solid-phase post-column reactor.** We dry-packed zinc particles into a 20 mm × 3.9 mm (i.d.) stainless-steel column equipped with 0.5-μm frits, then placed the column "in-line" between the chromatography column and the fluorometer.

**Results and Discussion**

Reduction of Vitamin K₁

Fluorometric detection of vitamin K₁ requires post-column reduction of the quinone to its fluorescent hydroquinone. Langenberg and Tjaden (12) developed a fluorescence liquid-chromatographic method for vitamin K₁, using a post-column electrochemical cell to reduce the vitamin. We experienced difficulties with this method, and delays in the analysis of plasma samples, because the reactor cell became contaminated. We also observed that the method suffers from low coulometric efficiency (60%) for the reduction of vitamin K₁ in the electrochemical cell. Also, when oxygen is present in the system, the coulometric efficiency is further diminished, and the fluorescence response is decreased by quenching. To overcome the problems with oxygen, we installed a column of metallic zinc particles to remove oxygen from the system as described by MacCrehan and May (14). Use of this zinc oxygen-scrubber column not only increased the fluorescence signal; it also resulted in near quantitative reduction of the vitamin at lower potentials than previously possible. We found with this system that vitamin K₁ can be quantitatively reduced to the hydroquinone in the presence of zinc and zinc ions. This finding validated our replacing the electrochemical cell with the column of metallic zinc. Theoretical details and performance characteristics of the zinc reactor column are described elsewhere (manuscript submitted to J Chromatogr).

The post-column reactor consists of zinc metal particles, which are dry-packed into 20 mm × 3.9 mm (i.d.) columns and inserted between the analytical column and the fluorometer. We found ideal performance at a flow rate of 1.0 mL/min, with use of the above-described mobile phase. Figure 1 shows a representative chromatogram for standards of vitamin K₁, vitamin K₁-epoxide, and dihydrovitamin K₁ (internal standard).

**Assay of Vitamin K₁ in Plasma**

Because of the very low concentration of vitamin K₁ relative to the high concentration of contaminating lipids present in the initial hexane extract of plasma samples, we were not able to detect vitamin K₁ by direct liquid chromatography of the lipid extract by either adsorption or reversed-phase chromatography. To obtain a bulk separation of non-polar lipids in plasma samples, we used a conventional solid-phase extraction technique with adsorption chromatography (9). In this step a non-polar fraction containing vitamin K₁ was eluted early from the column and thus was promptly resolved from more-polar lipids, which were retained by the silica.

![Figure 1. Separation of vitamin K₁ compounds by reversed-phase liquid chromatography on Hypersil-ODS](image-url)

Peak 1, K₁-epoxide (1.00 ng); 2, K₁ (0.50 ng); 3, K₁(H₂) (0.50 ng)
In the second step, the less-polar lipid fraction was further purified by a "reductive extraction" procedure. The principle of this methodology is based on our earlier observations that vitamin K₁ is quantitatively reduced to vitamin K₂ hydroquinone with zinc metal in the presence of zinc ions. Because vitamin K₁ hydroquinone is more polar than vitamin K₂, the hydroquinone can be selectively extracted into acetonitrile from hexane. Thus it was possible selectively to re-extract vitamin K₁ from the bulk of nonpolar acetonitrile-insoluble lipids present in the fraction obtained after chromatography on silica, by changing the polarity of the vitamin (i.e., by forming the hydroquinone of vitamin K₁).

Preliminary experiments with plasma samples suggested that most of the contaminating lipids remained behind in the hexane layer, while vitamin K₁ could be selectively extracted into the acetonitrile phase of the reductive extraction mixture. We investigated the recovery and reproducibility of this procedure for the routine assay of vitamin K₁ in plasma samples.

In initial experiments, tritium-labeled vitamin K₁ was dissolved in 1.0 mL of hexane to which 4.0 mL of the reductive extraction mixture was added. In similar control experiments, 4.0 mL of reductive extraction mixture containing no zinc metal was added to 1.0 mL of hexane containing radioactive vitamin K₁. The results demonstrated that although 30% of the quinone could be solubilized into acetonitrile in the absence of zinc metal, 70 to 80% of the radioactivity could be solubilized into the acidic acetonitrile reductive extraction mixture in the presence of zinc metal. Similar results were also obtained when plasma samples were enriched with radioactive vitamin K₁ and subjected to reductive extraction. When we subjected plasma samples to a second reductive extraction, only an additional 4% of vitamin K₁ was extracted into acetonitrile. Guided by these results, we incorporated the procedure into the routine assay for vitamin K₁ in plasma. The major advantage of incorporating this extraction procedure into the vitamin K₁ assay is that it exploits the acetonitrile-insolubility of the contaminants in the lipid extract (obtained after silica chromatography) as a sample-purification step, so that the fractionation step by absorption liquid chromatography used in previous assays for vitamin K₁ can be omitted.

Vitamin K₁ was resolved from the remaining impurities and internal standard by isocratic reversed-phase liquid chromatography on a column of Hypersil-ODS. This resolution was achieved by using the same chromatographic conditions described for the standards. Representative chromatograms for the assay of vitamin K₁ in plasma samples after post-column reduction to vitamin K₁ hydroquinone and fluorometric detection are shown in Figure 2.

Evaluation of Peak Identity and Purity

The validity of the assay depends on the complete resolution from interfering fluorescent peaks, so we established that the peaks measured contained only the pure compounds of interest. A good indication of peak purity can be obtained by comparing the fluorescence ratio of the height of the peak corresponding to vitamin K₁ at two or more specific excitation wavelengths while keeping the emission wavelength constant. For example, peak heights of vitamin K₁ were determined at 320 nm and 280 nm in both a plasma sample and an appropriate standard solution containing vitamin K₁. The ratio of the peak heights for plasma samples at two wavelengths could be compared with the

![Figure 2](image.png)

**Fig. 2.** Reversed-phase chromatograms for the assay of vitamin K₁ in (a) pool plasma, (b) individual plasma sample.

Peak 1, K₁; 2, dihydro K₁, the internal standard.

ratio obtained at the same pair of wavelengths for a standard solution of vitamin K₁. Table 1 illustrates the good agreement between the two.

**Table 1. Peak-Purity Test**

<table>
<thead>
<tr>
<th>Excitation wavelengths, nm</th>
<th>Vitamin K₁, standard</th>
<th>Pooled plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>320/340</td>
<td>2.08</td>
<td>2.08</td>
</tr>
<tr>
<td>340/280</td>
<td>0.74</td>
<td>0.78</td>
</tr>
</tbody>
</table>

*Fluorescence ratios shown were calculated by dividing the values for the two peak heights for vitamin K₁ obtained from separate chromatographic runs of pooled plasma at two different excitation wavelengths, with the emission wavelength constant kept constant at 420 nm.

**Table 2. Mean Vitamin K₁ Concentrations in Plasma from Fasting Subjects**

<table>
<thead>
<tr>
<th>This study</th>
<th>Shearer et al. (8)</th>
<th>Ueno and Suttle (11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vitamin K₁ concn., μg/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d</td>
<td>0.43 (6)</td>
<td>0.30 (15)</td>
</tr>
<tr>
<td>g</td>
<td>0.70 (16)</td>
<td>0.22 (15)</td>
</tr>
<tr>
<td>Total</td>
<td>0.56 (22)</td>
<td>0.26 (30)</td>
</tr>
</tbody>
</table>

| Values in parentheses are number of subjects. Mean for pooled plasma 0.50 (n = 15).
The "within-run" coefficient of variation for 12 replicate analyses of pooled plasma containing only endogenous vitamin K$_1$ was 6.0% and gave a mean value of 0.50 µg of vitamin K$_1$ per liter. For the same pool of plasma, the "between-run" CV for replicates was 12%. The "within-run" CV for the analysis of individual plasma samples (n = 40) was 5%, the mean value for vitamin K$_1$ being 0.64 µg/L. These results are summarized in Table 3.

Detection of Vitamin K$_1$ Epoxide

Our previous work with zinc columns suggested that vitamin K$_1$ epoxide could also be detected fluorometrically after reduction with zinc metal to vitamin K$_1$, followed by a further reduction to vitamin K$_1$ hydroquinone. We explored the possibility of detecting low endogenous concentrations of this metabolite of vitamin K$_1$ in plasma. For the assay of vitamin K$_1$ epoxide, we extracted two 2-mL plasma samples with hexane and omitted the reductive-extraction step. The lipid obtained after silica chromatography of the hexane extract was further fractionated by adsorption liquid chromatography as described previously (5, 6). When the equivalent of 2 mL of plasma was injected onto reversed-phase columns after collecting the appropriate fraction containing vitamin K$_1$ and vitamin K$_1$ epoxide from the adsorption liquid chromatography step, a peak corresponding to the retention time of vitamin K$_1$ epoxide was detected (Figure 3a). This peak represented about 30 pg of vitamin K$_1$ epoxide in 2.0 mL of plasma. When this same plasma pool was supplemented with 50 pg of vitamin K$_1$ epoxide and analyzed, a significant increase for the K$_1$ epoxide peak was observed (Figure 3b). These preliminary results (n = 2) suggest that the concentrations of K$_1$ epoxide in a sample of pooled plasma from fasting subjects may be about 15 ng/L. In a similar experiment, when a 2.0-mL aliquot of pooled plasma (n = 3) from subjects on anticoagulant therapy (warfarin) was analyzed and the equivalent of 1.0 mL of plasma was injected "on column," a peak with a retention time corresponding to that of K$_1$ epoxide was also detected (Figure 3c).

These studies suggest that the ratio of vitamin K$_1$ epoxide to vitamin K$_1$ in pooled plasma from fasting subjects and pooled anticoagulated plasma is 0.16 (n = 2) and 0.96 (n = 3), respectively.

We identified the peak corresponding to putative vitamin K$_1$ epoxide in a similar manner to that described for vitamin K$_1$. The wavelength ratios we obtained identified vitamin K$_1$ epoxide in anticoagulated plasma.

An objection to use of the reduction-extraction procedure as a sample-purification step is that vitamin K$_1$ epoxide is converted to vitamin K$_1$ during this procedure. However, when plasma samples were concurrently processed by the previously described methods (5, 6) and the reductive extraction procedure, we obtained similar values for vitamin K$_1$ (data not shown). Furthermore, the value determined for vitamin K$_1$ epoxide in pooled plasma suggests that vitamin K$_1$ epoxide would make an insignificant contribution to the peak height observed for vitamin K$_1$ and would have no notable effect on the accuracy of the assay procedure. For example, during the reversed-phase liquid-chromatographic assay for vitamin K$_1$ in plasma, an equivalent of 0.4 mL of plasma was injected "on-column." From the concentration of K$_1$ epoxide determined in pooled plasma, the peak for K$_1$ would be enhanced by 6 pg/0.4 mL by the epoxide, an amount of vitamin K$_1$ that would not be detected fluorometrically.

The use of the reductive extraction step can only be justified, however, if no anticoagulant drugs (which cause K$_1$ epoxide to accumulate in plasma) have been administered to subjects. If anticoagulated subjects are to be monitored, the additional step, chromatography on silica, must be incorporated into the assay, to replace the reductive extraction.

Absorption of Vitamin K$_1$

In studying the absorption and metabolism of vitamin K$_1$ in humans it would be useful to be able to measure the vitamin directly in plasma. Figure 4 shows the time course for the appearance of vitamin K$_1$ in plasma, as determined by our procedure, after a subject ingested 100 µg of vitamin K$_1$. These results agree with previously reported data on the absorption of vitamin K$_1$ and show the feasibility of measur-

---

**Table 3. Precision of the Present Assay**

<table>
<thead>
<tr>
<th>Plasma Type</th>
<th>Mean vitamin K$_1$ concn, µg/L</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individual</td>
<td>0.64</td>
<td>5.0</td>
</tr>
<tr>
<td>Pool</td>
<td>0.50</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.0</td>
</tr>
</tbody>
</table>

---

**Fig. 3. Detection of vitamin K$_1$ epoxide in:**

- (a) 2.0 mL of preprandial plasma
- (b) 2.0 mL of preprandial plasma supplemented with K$_1$ epoxide
- (c) 1.0 mL of pool plasma from subjects taking anticoagulants (warfarin)

Peak 1, K$_1$ epoxide; 2, K$_1$.

---

**Fig. 4. Vitamin K$_1$ as measured in plasma of a subject after an oral 100-µg dose of vitamin K$_1$.
ing low concentrations of vitamin K₁ in plasma in clinical situations, to monitor its absorption.

Several major advantages are realized with the present assay. Use of the electrochemical cell for reduction of the vitamin is obviated, which minimizes equipment required and eliminates cell-contamination problems. Because of the increased reduction efficiency of the zinc column as compared with the electrochemical cell, detection limits for vitamin K₁ are decreased; thus a smaller sample is needed for analysis. Finally, the zinc column also allows for the reduction of the major metabolite of vitamin K₁, vitamin K₁ epoxide, which is not reduced by the electrochemical cells. Because vitamin K₁ epoxide accumulates during anti-coagulant therapy with coumarin, determination of this metabolite may prove to be important in the management of such therapy. Further improvements in sample preparation should move the analysis of vitamin K₁ from the research laboratory into the clinical setting.

References