Analysis of Supplemented Vitamin K₁(20) in Serum Microsamples by Solid-Phase Extraction and Narrow-Bore HPLC with Multichannel Ultraviolet Detection

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A sensitive method for the determination of vitamin K₁(20) in serum microsamples (50 μL) has been developed, utilizing solid-phase extraction with C₁₈ Bond-Elut columns and reversed-phase narrow-bore high-performance liquid chromatography [2.1 mm (i.d.), column] with a nonaqueous eluent. Recovery from serum (49 ng/mL) was 76% (n = 2). Peak homogeneity was assessed by photodiode array detection with absorbance ratio, spectral normalization, and transformation to the first- and second-derivative chromatograms. Calibration data at 248 nm over two ranges (20–200 ng/mL, 200–4000 ng/mL) varied linearly with concentration and were suitable for studies of vitamin K₁ supplementation. By comparison with conventional columns, sensitivity was increased twofold.

Additional Keyphrases: hemorrhagic disease of the newborn · multichannel detection · assessment of peak homogeneity · pediatric clinical chemistry

Hemorrhagic disease of the newborn, a bleeding disorder occurring in the first eight weeks postpartum, is characterized by a deficiency of the four vitamin K-dependent clotting factors: II (prothrombin), VII (proconvertin), IX (thromboplastin), and X (Stuart–Prower factor). Synthetic vitamin K ("Konakion"; Roche Products Ltd., Welwyn Garden City, Hert., U.K.) is commonly administered prophylactically to the newborn infant to prevent hemorrhagic disease, the recommended dose being 1 mg by intramuscular injection. Whether all newborn infants are deficient in vitamin K is still controversial (3).

HPLC methods for quantifying K vitamins in plasma or serum can be divided into two general groups. In one, to overcome the problem of co-extracted ultraviolet-absorbing contaminants (mainly lipids), which pose a major problem in the detection of vitamin K₁ in plasma/serum, "multidimensional" chromatography is used (4–11). Sample pretreatment by liquid–liquid extraction is followed by further separation adsorption chromatography, then by a second chromatographic stage involving reversed-phase conditions (hence the term "multidimensional"). Detection modes have included ultraviolet (4–5, 7, 9) and electrochemical (6, 10, 11), the most sensitive being electrochemical detection [20 pg/mL was reported by Hart et al. (11)]. Other types of multidimensional chromatography involve the use of two detection systems (12–14).

The high level of sensitivity of these multidimensional techniques is achieved by using relatively large volumes of serum (often 10 mL). Multidimensional chromatography is also labor intensive, expensive, and, because of the large sample requirement, unsuitable for routine monitoring in pediatrics.

The second group of methods is simpler to use and is adequate for therapeutic monitoring of K₁ concentrations after supplementation with synthetic K₁, either orally or intramuscularly (2), or for measuring pharmacological concentrations of K₁ in rabbit plasma (15). However, these methods lack the requisite sensitivity for the measurement of endogenous amounts of the K vitamins. Generally, they combine liquid–liquid extraction and a single column (reversed-phase or normal-phase) chromatography with ultraviolet detection.

McNinch et al. (1), using a method previously developed (7), determined plasma concentrations of K₁ in 107 neonates after oral or intramuscular administration of 1 mg of vitamin K₁. In babies given the vitamin orally at birth or orally with the first feeding, the peak median concentration (73 ng/mL) occurred 4 h afterwards. Concentrations in plasma after intramuscular injection exceeded those in the oral group, giving a peak median concentration of 1781 ng/mL by 12 h, decreasing to 444 ng/mL at 24 h. Lucock et al. (2), with fewer subjects (26), found values of the same order.

In the procedure proposed here, we use a simple solid-phase extraction process with an octyl-bonded silica sorbent, in combination with narrow-bore liquid chromatography (LC), which lowers the limit of detection (LOD) to about half that of a conventional column system. To evaluate the homogeneity of the eluted peaks, we examined rapid-scanning linear photodiode array (LDA) detection.

Materials and Methods

Reagents and Materials

Acetonitrile, isopropanol, dichloromethane, and methanol (all HPLC grade; Rathburn Chemicals, Walkerburn, Lothian, U.K.) were used as received. Glass-distilled water and all mobile phases were filtered under reduced pressure through 0.45-μm pore-size MA filters (Millipore Corp., Bedford, MA) with an all-glass apparatus. All volumes used in the preparation of mobile phases were measured separately. Degassing, as required, was effected with a stream of helium, purging at 180 mL/min for 5 min, then in a continuous stream of 5 mL/min.

Vitamins K₁(20) (phylloquinone) and K₃, 2,3-epoxide were donated by Hoffmann-La Roche, Basle, Switzerland. Vitamins K₁(25), cis-K₁(20), K₃(20), K₃(30), and K₃(35), originally supplied by Hoffmann-La Roche, were a gift from the Paediatric Research Unit, Leeds General Infirmary, Leeds, U.K. Human serum from a volunteer, known not to be taking any medication, was obtained from the Haematology Department, St. Luke’s Hospital, Bradford, U.K.

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Standard solutions. The vitamins K₁(20), cis-K₁(20), K₂(25), K₂(23), epoxide, K₂(20), K₃(30), and K₄(35) were weighed on a Cahn C-31 microbalance (Cahn Instruments, Cerritos, CA). Standards for the calibration curves were prepared by dilution in HPLC mobile phase. Vitamin K₁(25), a synthetic analog of vitamin K₁(20), was selected as the internal reference standard (Figure 1). Low-acetin glassware, rinsed with acetone to prevent alkali degradation of the vitamins, then dried, was used for all solutions and Parafilm was wrapped around each sealed container. In most cases aluminum foil was also wrapped around each vessel as a further precaution. All solutions were stored in the dark at -20 °C.

Serum standards were prepared by mixing standards gently at various dilutions into human serum (16). Small aliquots of standards were mixed into larger volumes of serum (usually 50 µL of standard in HPLC mobile phase made up to 1000 µL with serum). This small amount of mobile phase (about 5% of the final volume) did not cause any noticeable protein denaturation. Incorporation of vitamin K₁ into the lipoproteins was assumed to be complete after 2 h (16). The vials were protected from light during and after this process and all glassware was rinsed with acetone and dried. The samples were stored in the dark in a refrigerator, although most supplemented serum samples were discarded at the end of each day.

Sample Pretreatment

Vitamin K circulates in the blood in association with β-lipoproteins. Any extraction procedure should therefore effectively disrupt the lipoprotein structure, leading to the liberation of the associated lipids. A Sep-Pak silica cartridge method (17) has been used after extraction of vitamin K₁ from plasma with hexane after deproteinization with ethanol. Here we propose a simple solid-phase extraction process for small samples. We have found C₈ (octyl) columns to be satisfactory, allowing optimum retention and elution of the vitamins K₁(20) and K₂(25).

We conditioned the 1-mL C₈ column with 2 mL of methanol and 1 mL of distilled water, then loaded 50 µL of a supplemented serum sample directly onto the sorbent bed; we then added, similarly, 30 µL of vitamin K₂(25) in mobile phase: acetonitrile/isopropanol/dichloromethane (70/10/20, by vol). About 1 min later, we filled the Bond-Elut column with distilled water (about 1 mL), then washed it with two further column volumes of distilled water. After drying the column (removing the liquid by suction), we applied 150 µL of mobile phase to the column and allowed this to permeate the column bed for 1 min before collecting the eluent under reduced pressure. Another 150 µL of mobile phase was applied similarly. The combined (300 µL) eluate was then evaporated under nitrogen for a few minutes, with use of a Reactivap unit (Pierce UK, Cambridge, U.K.), and the residue was reconstituted in 50 µL of mobile phase. We injected 10 µL of this onto the chromatograph by filled-loop injection.

Chromatography

The analytical columns were 100 × 2.1 mm i.d. (narrow-bore) and 100 × 4.6 mm i.d. (regular). The narrow-bore column was packed with the reversed-phase fully-capped Spherisorb 5-µm RP-18 ODS-102 (Brownlee, Santa Clara, CA). This cartridge column from the Brownlee MPLC system was used in the main part of the work in conjunction with a guard column (30 × 2.1 mm i.d.: ODS-032; Brownlee) of the same material. The regular-bore column was packed "in-house" with reversed-phase ODS-2 5-µm Spherisorb (Phase Sep, North Queensferry, U.K.). Spherisorb, a silica with totally porous, spherical particles, is fully capped, with very few residual silanol groups, making it suitable for nonaqueous reversed-phase chromatography. Using the narrow-bore analytical and guard columns, a Rheodyne injection valve (Berkeley, CA; Model 7125, fitted with a calibrated 10-µL loop, and an LKB 2150 pump (LKB-Produkter AB, Bromma, Sweden), we investigated two types of detection systems.

Detection system IA was the single-channel variable-wavelength LDC SpectroMonitor 3000 Detector (LDC/Milton Roy, Stone, Staffordshire, U.K.), fitted with a 1-µL flow cell, 3-mm pathlength. This was used at an optimized detection wavelength of 248 nm. The potentiometric chart recorder used was from LKB (Model 2210).

Detection system IB was the Hewlett-Packard (HP) multichannel LDA detector (Model 1040A; Hewlett-Packard, Waldbronn, F.R.G.), fitted with a 4.5-µL flow cell, 6-mm pathlength, and used with a spectral resolution of 2 nm per diode in the range 200–400 nm. The 1040A detector was connected via an HP-IB interface bus (IEEE 488) to an HP Chem Station 300 9000 with a Model 9133 disc unit containing a 20-megabyte hard disc and 3.5-in. floppy disc drive; an HP 7470A plotter; and an HP 2225A "think-jet" printer.

The regular column, a 20-µL Rheodyne injection valve (also Model 7125), and the LKB 2150 pump were used with detection system II. This system comprised the single-channel variable-wavelength LDC SpectroMonitor 3000 detection system fitted with a 14-µL flow cell, 10-mm pathlength. Again, we used this system at the optimized detection wavelength of 248 nm.

Other Apparatus

Connecting tubing (0.13 mm i.d.) and in-line filters (Model 7315 with 0.5-µm frits and a 1.5-mm-diameter filter disc) were supplied by Rheodyne. We also used a Model 1702 electronic balance (Sartorius GmbH, Göttingen, F.R.G.), a Spiramix Model 5 vortex-type mixer (Spiramix, Denley, Sussex, U.K.), and a microcentrifuge (Model 320; Quickfit Instrumentation, Stone, Staffordshire, U.K.). Nitrogen was supplied by BOC (Guildford, Surrey, U.K.); the ReactiTherm stirring/heat unit (part no. 18971), block (no. 18802), and the Reactivap evaporating accessory (no. 18780) were from Pierce UK.

For the solid-phase extraction work, we operated a Vac-
Elut manifold (Analytichem International, Harbor City, CA) at various pressures. All samples were extracted with Bond-Elut (Analytichem International) solid-phase disposable extraction columns (100 mg of sorbent, 1-mL column volume) packed with C8 sorbent.

Preliminary experimentation established that the optimum mobile phase, in terms of peak shape and N, for detecting K vitamins, was a tertiary mobile phase similar to the system employed by Lucock et al. (2); acetonitrile/isopropanol/dichloromethane (68.5/22.2/9, by vol) with ultraviolet detection at 270 nm.

We found that slightly better results were obtained by maintaining the isopropanol concentration at 100 mL/L, varying the dichloromethane and acetonitrile, and monitoring at 248 nm. The optimized solvent system was acetonitrile/isopropanol/dichloromethane (70/10/20, by vol). After evaluating several of the K vitamins, we decided vitamin K1 (25) was the most suitable internal standard. For narrow-bore work, the optimized solvent gave acceptable resolution of vitamin K1(20) (k' = 2.17) and of the internal standard, vitamin K1(25) (k' = 4.04), resulting in a total analysis time of 13.5 min at a flow rate of 0.10 mL/min (Figure 2). N was >36,000 per meter for each peak, and the resolution factor (Rk) was 3.07 for vitamin K1(20) and internal standard.

Natural K1 exists only as the trans-isomer, whereas synthetic K1 contains a mixture of 70% trans-30% cis-isomers at the 2,3 position of the phytyl side chain. Under these optimized chromatographic conditions the cis- and trans-isomers were co-eluting, as determined by injecting the pure cis-isomer and comparing its elution time and k'. The cis- and trans-isomers are usually separated only by normal-phase chromatography.

**Results**

The narrow-bore chromatographic procedure with solid-phase extraction produced well-resolved and reproducible peaks. For quantifications, we used peak-height response ratios with respect to the internal reference standard (IS) (Figure 2). Two sets of calibration data were prepared to deal with two methods of administration: 198–3960 ng/mL and 20–198 ng/mL. The figures of merit for each of these calibration curves at 0.02 A full scale and 0.001 A full scale, respectively, are presented below.

For the calibration data for sera supplemented with high concentrations of vitamin K1(20), over the range of interest (198–3958 ng/mL), peak-height response ratios were linear: y = 0.0002x - 0.0003; r = 0.9996 (n = 5).

For the lower concentration calibration data, peak-height response ratios were again linear over the range of interest (20–198 ng/mL): y = 0.0092x + 0.0100; r = 0.9984 (n = 6).

Analytical recoveries, assessed by comparison of peak heights with those obtained from directly injected standard mixtures, are summarized in Table 1. At 198 ng/mL, recovery was 80.5%; at 49 ng/mL, it was 76.5%. The within-batch CV for vitamin K1(20) was 4.27% at 198 ng/mL (n = 4).

**Detection Limits**

We adopted the conventional definition of sensitivity (LOD, as weight injected on-column) as a signal-to-noise ratio of 2. A comparison of the LOD of the two single-channel systems, IA and II, having the 100-mm narrow-bore column and 100-mm regular-bore column respectively, showed 1.7 times greater sensitivity for system IA: 109 pg vs 181 pg (system II). This increase is not, however, the maximum increase (a factor of 4.8) predicted from theory (18).

Using the narrow-bore column system, extracting a 50-μL serum sample, reconstituting in 50 μL of mobile phase, and assuming an extraction efficiency of 80%, we calculate that this LOD corresponds to a concentration of 14 ng/mL for a 10-μL injection volume. Extracting a 500-μL serum sample and reconstituting in 50 μL of mobile phase would correspond to a concentration of 1.4 ng/mL because of pre-concentration. These concentrations would allow quantification of supplemented concentrations found in adults (in a minimum of 500 μL of serum), but would not allow measurement of endogenous concentrations in neonates.

**Peak Homogeneity by Multichannel Detection**

After the narrow-bore column with solid-phase extraction procedure was developed, we used a LDA ultraviolet rapid-scanning detector to examine the results in more detail.

The (A, λ, t) matrix or spectrochromatogram generated by these detectors can be used to examine the purity of chromatographic peaks (Figure 3). Data can be examined to obtain the elution profiles of a sample at any wavelength within the range, or to present the spectrum of each component for qualitative comparison with standards. The three-dimensional (3-D) spectrochromatogram presented in Figure 3 illustrates the use of spectral data acquired at 1-s intervals for examination as to whether any peaks other than those anticipated are present. The 3-D plot, and spectra taken from this, confirm the choice of 248 nm as the optimum wavelength of detection.

Peak purity is a central question in bioanalytical studies.

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**Table 1. Analytical Recovery of Vitamin K1(20) from 50-μL Supplemented Serum Samples**

<table>
<thead>
<tr>
<th>Conc, ng/mL</th>
<th>Recovery, %</th>
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<tbody>
<tr>
<td>3959</td>
<td>82.1</td>
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<tr>
<td>990</td>
<td>86.4</td>
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<tr>
<td>198</td>
<td>80.5</td>
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<td>99</td>
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<td>49</td>
<td>76.5</td>
</tr>
<tr>
<td>40</td>
<td>78.4</td>
</tr>
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</table>
The additional dimension of wavelength generated by the LDA has led to the development of several mathematical methods to address this problem. The absorbance ratio technique (19) identifies co-eluting impurity peaks as a distortion of the constant square-wave produced for a pure compound. As can be seen for the absorbance ratio plots of both vitamin K1(20) and K1(25) in serum samples, no impurity was detected (Figure 4), although the "square-wave" did show some disturbances. This technique is of use only when the absorbance ratio of the impurity, at the same two wavelengths, differs from that of the main component. The LDA can be used to aid the process of selecting wavelength pairs when the nature of the impurity is unknown (20).

Spectral normalization (Figure 5) is a simple technique whereby spectra recorded at various points (usually upslope, apex, and downslope) can be retrieved and normalized to the apex of the peak. The normalized spectra can then be visually inspected for peak homogeneity. As shown in Figure 5, there was no inhomogeneity of the peaks in the serum samples.

First and second derivatives of zero-order chromatograms transform shoulders and peaks into sharpened peaks, allowing more points of comparison (Figure 6). The first and second derivatives of the K vitamins in serum samples again confirm peak purity. The derivative techniques require a minimum peak separation, and interference by satellite artifacts often limits their usefulness (21).

Fig. 3. Spectrochromatogram of a serum sample with added K1. For pretreatment process, chromatographic conditions, and detector apparatus, see text. Time window 7.00-14.90 min. Peaks: 1, K1(20), 4 μg/mL; 2, K1(25), 10 μg/mL.

Fig. 4. Absorbance ratio plots with \( \lambda_s = 270 \text{ nm} \) and \( \lambda_p = 275 \text{ nm} \), confirming the purity by "square wave" of both (I) vitamin K1(20) and (II) vitamin K1(25).

Fig. 5. (I) Normalized upslope (U), apex (A), and downslope (D) spectra of vitamin K1(20) peak in serum; (II) normalized apex spectra of vitamin K1(20) peak in standard and in serum samples.

Fig. 6. First (I) and second (II) derivative resolution enhancement of chromatogram in Fig. 4 (Bottom).
Thus, the LDA enables confirmation of peak purity and allows close examination of the clean-up of the solid-phase extraction process.

Discussion

The lipophilic nature of the K vitamins puts a high demand on their chromatographic separation from fat-soluble co-extractants. There is usually an unfavorable ratio of the amount of K, to the other lipids extracted from the biological material. The problem of determination is further complicated by their very low endogenous concentrations.

More recently, Shearer et al. (7) have shown that the concentration of vitamin K in plasma correlates with that of lipids in plasma. In patients with hyperlipoproteinemia, the plasma K concentration appeared to be significantly higher than in healthy subjects (range 0.16 to 12.75 ng/mL, with a mean value of 1.16 ng/mL). The K vitamins are insoluble in water and are not excreted in the urine.

The method we describe is rapid, and samples can be analyzed by parallel extraction in batches of six on the Bond-Elut vacuum manifold. Combined with an analysis time of 13.5 min for the nonaqueous reversed-phase chromatography, the total analysis time is <20 min per sample. Compared with other reported methods involving multidimensional liquid chromatography and requiring serum sample volumes of 2–10 mL (4–14), this method has better sample size requirements, speed, and sensitivity. Even in comparison with the method of Lucocq et al. (2), who used 250 μL of sample and reported a detection sensitivity of 15–20 ng/mL, the used method compares well.

The extraction process does, however, require validation with real serum samples, because the process of adding standards to serum may not produce a true picture of the vitamin K, association with β-lipoproteins. Langenberg (16), whose method we followed for preparing the serum standards, found that about 50% of vitamin K, was extracted from hexane into serum by gentle mixing for 10 min, indicating that the vitamin would be incorporated into the lipoproteins in the method we described. The serum we used was from one subject only, in which no vitamin K was detected.

The maximum sample we applied to the Bond-Elut column was 500 μL, in which we detected as little vitamin K, as 1.4 ng/mL (reconstituting the sample in 50 μL of mobile phase). To measure the lower endogenous concentrations in neonates, we would have to use a more sensitive mode of detection such as electrochemical or fluorescence detection. However, the use of electrochemical detection (e.g.) would require a more aqueous mobile phase.

The method could, however, be used to measure vitamin K, in serum after supplementation with Konjakon; in investigations into the calcification process of the bone and in disorders of the bone, e. g., in osteoporotic patients (11) and in thromboembolic disease. Vitamin K, is also administered to adults with fat malabsorption, e.g., in obstructive jaundice.

The solid-phase extraction method is specifically designed for injection of small aliquots (≤10 μL) onto the chromatograph. For repeat injections on a conventional column, e.g., 20-μL filled-loop injections, the sample would have to be reconstituted in a larger volume of mobile phase, decreasing the sensitivity of the extraction process for small serum samples. During chromatographic analysis the further advantage of solvent economy is an important bonus of the narrow-bore system.

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References