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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF VITAMIN K IN HUMAN SERUM

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SUMMARY

A sensitive combined adsorption and reversed-phase high-performance liquid chromatographic procedure is described that permits the determination of endogenous vitamin K_1 levels in serum.

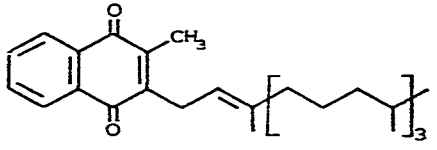
The separation of vitamin K_1 (*cis-* + *trans*-isomers), vitamin K_1 epoxide and the menaquinones MK-2, MK-4 and MK-9 by adsorption, reversed-phase and cyano-bonded phase chromatography is discussed. The methodology was further developed for the quantitative measurement of vitamin K_1 in human serum. Concentrations as low as 500 pg/ml in serum could be detected. The identity of the vitamin K_1 peak was confirmed by UV absorption spectrophotometry and re-chromatography. The method has been applied to the determination of serum levels in normal healthy individuals and patients treated with vitamin K_1.

INTRODUCTION

There is a growing interest in the role, biochemical function and metabolism of vitamin K in man. It is well known that this vitamin is essential for the formation of prothrombin and other blood-clotting proteins by the liver. During the last few years, much progress has been made in understanding the biochemical mechanism involved[1]. Recent investigations indicate that vitamin K would also be required for the synthesis of other calcium-binding proteins[2]. Its role in cell respiration was associated with possible anti-tumoral activity[3,4]. As there is no direct and practical method available for the measurement of vitamin K, prothrombin levels are determined as an indication of the vitamin K status. However, hypoprothrombinemia may

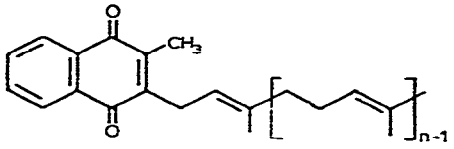
also result from severe liver disease, such as cirrhosis, not associated with vitamin K deficiency. Studies on the biochemistry and function of vitamin K also require a specific determination of the various forms of the vitamin and its metabolites.

The two naturally occurring and biologically most important forms of vitamin K are phylloquinone or vitamin K_1 (I) from plant origin and the menaquinones or vitamin K_2 series, synthesized by bacteria (II). The length of the isoprenoid side-chain in menaquinones is defined by its carbon number, or the number of isoprenoid units, *e.g.*, vitamin $K_{2(20)}$, or menaquinone-4 (MK-4). After fractionating an extract of 5 kg of human liver by classical column and thin-layer chromatography, Duello and Matschiner[5] obtained mass spectrometric evidence for the presence in human body of both phylloquinone (K_1) and some menaquinones (MK). This supports the hypothesis that the vitamin K status is determined by food intake (phylloquinone) and intestinal synthesis (menaquinones).



Phylloquinone

I



Menaquinone - n

II

Phylloquinone 2,3-epoxide (K_1 epoxide) was found as a metabolite after the administration of radioactively labelled vitamin K_1 (ref. 6). On the other hand, the formation in the liver of MK-4 from other vitamin K compounds has also been reported[7,8].

The gas chromatography of phylloquinone (K_1) and menaquinones up to MK-9 has been described[9]. However, in spite of the use of short columns, low stationary phase loads and high analysis temperatures, retention times of 30 min and more are common. Vetter *et al.*[10] suggested reduction to the naphthohydroquinone, followed by silylation to prevent on-column degradation of the vitamin. The use of this procedure was criticized by Dialameh and Olson[11].

In our opinion, high-performance liquid chromatography (HPLC) appears to be a more appropriate approach for these unstable high-molecular-weight compounds. Absorptiometric detection is indicated as both phylloquinone and the menaquinones strongly absorb UV radiation [for K_1, $\varepsilon = 20{,}300$ at $\lambda_{max.}$ (248 nm)]. Elliot *et al.*[12] used HPLC to determine vitamin K_1 and vitamin K_1 epoxide added to liver homogenates. While our work was in progress, papers appeared describing the separation of K_1, K_1 epoxide and MK homologues in standard solutions and injec-

tions[13–15]. Bjornsson *et al.*[16] followed radioactively labelled K_1 and its epoxide in human plasma by HPLC. In their work it was necessary to add the radioactive tracer to obtain sufficient sensitivity to measure these compounds at physiological concentrations.

Our aim was the development of a method selective and sensitive enough to determine endogenous levels of vitamin K. The separation of phylloquinone, phylloquinone 2,3-epoxide and the menaquinones by adsorption, reversed-phase and cyano-bonded phase chromatography was studied. The eluent composition and column dimensions were optimized so as to allow an optimal detection limit for these compounds in a serum extract. We fractionated the serum extract on a silica adsorption column and subsequently quantified the fractions containing compounds of interest on a 9% octadecyl reversed-phase column. The vitamin K peaks were well resolved from interfering co-extracted serum compounds with excellent sensitivity (for vitamin K_1, the detection limit is less than 500 pg/ml). This method was used for identification and determination of vitamin K_1 in human serum with [^{3}H]phylloquinone as an internal standard. Serum levels found in normal healthy individuals and in patients treated with vitamin K_1 are given. It is interesting that, independently of our work, a combined adsorption and reversed-phase HPLC system has recently been described by Thompson[17] for the determination of phylloquinone in milk.

EXPERIMENTAL

Reagents

Phylloquinone (K_1) was obtained from ICN Pharmaceuticals (Cleveland, Ohio, U.S.A.) and from Serva (Heidelberg, G.F.R.). Nuclear magnetic resonance (NMR) spectrometry was used to determine the ratio of *cis*- and *trans*-phylloquinone in the respective standards[18]. The menaquinones MK-2, MK-4 and MK-9 were a gift from Dr. Weber and Dr. Gloor (Hoffmann-La Roche, Basle, Switzerland) as well as [phytyl-1,2-^{3}H]vitamin K_1 (700 μCi/mg). The radiochemical purity of the latter was checked by thin-layer chromatography on silica gel with the solvent mixture light petroleum–diethyl ether (98:2) and radioscanning. We isolated *trans*-[^{3}H]phylloquinone after chromatography on a silica HPLC column (20 cm × 4.6 mm I.D.) eluted with 0.2% acetonitrile in *n*-hexane and used this to prepare the final internal standard solution. Phylloquinone 2,3-epoxide (K_1 epoxide) was synthesized according to Tishler *et al.*[19] and the chemical identity of the product was confirmed by mass spectrometry. All standard solutions were prepared in the eluent used in the respective HPLC procedure and stored at 5° in dark brown vessels. All solvents were of analytical reagent grade from Merck (Darmstadt, G.F.R.). The scintillation cocktail used was Insta-Fluor II from Packard (Downers Grove, Ill., U.S.A.).

Columns

The column dimensions were 10 cm × 2 mm I.D., 15 cm × 3.2 mm I.D. and 20 cm × 4.6 mm I.D. The following packing materials were obtained from RSL (St. Martens-Latem, Belgium): silica (Rsil, 5 μm); high-load (18% organic C) and low-load (9% organic C) octadecyl reversed-phase (Rsil C_{18} HL, 10 μm, and Rsil C_{18} LL, 5 μm and 10 μm); and a cyano-bonded phase (Rsil CN, 10 μm). LiChrosorb RP-8, 5 μm, was supplied by Merck. Silica columns were packed from a 10% slurry

in methanol with light petroleum (5000 p.s.i.) as pressurizing liquid. For bonded phases we used acetonitrile (6400 p.s.i.) and a 10% slurry, for C_{18} and CN in carbon tetrachloride–methanol (9:1) and for RP-8 in glycerol–methanol (1:4). The pre-column (5 cm × 4.6 mm I.D.) was dry packed with Perisorb A, 30–40 μm (Merck). The columns were operated at ambient temperature.

Apparatus

A Hewlett-Packard HP 1084A liquid chromatograph (Hewlett-Packard, Böblingen, G.F.R.) equipped with a fixed-wavelength UV detector (254 nm) was used for the fractionation of the serum extract on silica. The quantitative work was carried out on a second apparatus, consisting of a Varian 4100 LC pump (Varian, Palo Alto, Calif., U.S.A.), a Rheodyne injection valve with 20- and 150-μl loops (Rheodyne, Berkeley, Calif., U.S.A.) and a Pye Unicam LC 3 variable-wavelength detector (Pye, Cambridge, Great Britain).

Scintillation counting was performed on a Packard 3380 Tricarb liquid scintillation spectrometer.

Serum samples

For the developmental work on the assay, a pool of normal human serum was used. Normal ranges for vitamin K_1 were determined using serum freshly taken from apparently healthy adult laboratory workers. Serum samples from patients treated with vitamin K_1 were obtained through the kind help of Dr. F. Hublé, Internal Medicine Dept., Academic Hospital, Ghent. They were taken just before and 1 h after the administration of an i.m. dose of 10 mg of Konakion (vitamin K_1).

Methods

The following compounds were used as standards and chromatographed under various elution conditions on silica, high- and low-load octadecyl-, octyl- and cyano-phases: *cis*- and *trans*-K_1, K_1 epoxide, MK-2, MK-4 and MK-9.

Serum extracts were prepared from 2 ml of serum to which 5000 dpm of *trans*-[^{3}H]-K_1 in 40 μl of methanol were added as internal standard. Incubation of the serum with the labelled product at 4° for 60 min ensured a thorough equilibration of exogenous and endogenous K_1. The serum was deproteinized with 2 ml of methanol and extracted with 5 ml *n*-hexane on a vortex mixer for 2 min. After centrifugation (4200 *g*, 5 min) the supernatant fluid was transferred to a brown conical glass tube and evaporated to dryness on a rotating vacuum evaporator (Evapo-Mix, Büchler, Fort Lee, N.J., U.S.A.) at ambient temperature. The residue was dissolved in 200 μl of eluent, injected on top of a 20 cm × 4.6 mm I.D. silica column and eluted with 0.2% acetonitrile in *n*-hexane at a flow-rate of 2 ml/min. The fraction corresponding to vitamin K_1 was collected, evaporated to dryness under a stream of nitrogen and the residue redissolved in 100 μl of the eluent used for reversed-phase chromatography. An aliquot corresponding to 1 ml of serum was injected on an octadecyl low-load (RP-18 LL, 5 μm) 15 cm × 3.2 mm I.D. column and eluted with acetonitrile–dichloromethane (92:8) at a flow-rate of 1 ml/min.

The effluent was monitored with a variable-wavelength detector at 248 nm [sensitivity 0.005 absorbance unit full scale (a.u.f.s.)]. The fraction of reversed-phase effluent, corresponding to the K_1 peak, was collected in a scintillation vial. After

evaporation of the acetonitrile–dichloromethane mixture, the residue was redissolved in 8 ml of scintillation fluid and counted.

The extraction recoveries of vitamin K_1 from serum were determined by evaporating exactly 4.00 ml of the *n*-hexane layer directly in a counting vial and redissolving the residue in scintillation fluid. To compensate for quenching, the percentage counting efficiency was determined by recounting each vial after the addition of a known number of dpm of [^{3}H]toluene.

For the identification of the peak corresponding to vitamin K_1, an extract was made from 10 ml of serum without the addition of internal standard. After purification on the silica column, five fractions (each corresponding to 2 ml of serum) containing K_1 were pooled, evaporated to dryness under nitrogen and the residue was redissolved in 400 μl of reversed-phase eluent. Aliquots of exactly 20 μl (corresponding to 0.5 ml of serum) were injected on to the C_{18} LL reversed-phase column at different detector wavelength settings and the peak height of the compound of interest was plotted *versus* wavelength. A second confirmation was obtained by re-chromatography of the K_1 peak collected from the RP-18 LL column. The reversed-phase eluent was carefully evaporated on the Evapo-Mix and the sample redissolved in 100 μl of *n*-hexane. Aliquots of 50 μl were chromatographed on 15 cm × 3.2 mm I.D. silica and cyano-bonded phase columns.

RESULTS AND DISCUSSION

Adsorption chromatography on silica (20 cm × 4.6 mm I.D. and 15 cm × 3.2 mm I.D. columns) allowed the separation of the *cis*- and *trans*-isomers of K_1, K_1 epoxide and the lower (MK-2, MK-4) from the higher (MK-9) menaquinones (Fig. 1). As these substances are highly non-polar, eluents with very low eluotropic strengths (ε_0) were used. To avoid the difficulty of saturating apolar solvents (*e.g.*, *n*-hexane) with water, a moderator was used[20,21]. Mixtures of isopropanol, dichloromethane and acetonitrile with *n*-hexane or light petroleum were studied as eluents. A 0.2% solution of acetonitrile in *n*-hexane (or light petroleum) gave the best peak shape and compromise between resolution and analysis time [capacity ratio (k') of 9.5 for MK-9, Table I]. A greater selectivity of acetonitrile over dichloromethane was observed. *cis*-K_1, *trans*-K_1 and the epoxide were no longer resolved when 10% dichloromethane in *n*-hexane was used to elute the silica column (Table I). In general, the selectivity will be greater if the concentration of the stronger component of the solvent mixture is less than 5%[22]. Moreover, the use of dichloromethane resulted in unsymmetrical peaks, even when ternary acetonitrile–dichloromethane–*n*-hexane mixtures were used. A third reason to prefer acetonitrile over dichloromethane (and also *n*-hexane over light petroleum) was related to the collection procedure to be used for serum samples. When 2.5 ml of the 0.2% acetonitrile in *n*-hexane eluent were evaporated to dryness and the residue, redissolved in acetonitrile, was injected on to a reversed-phase column (RP-8 or RP-18), only a small solvent peak was observed, in contrast to the large signal obtained from dichloromethane or light petroleum residues.

Reversed-phase chromatography, whether on C_8, or C_{18} high-load or low-load columns, resulted in the separation, in order of elution, of MK-2, MK-4, K_1 epoxide, K_1 (*cis*- and *trans*-eluted as a single peak) and MK-9 (Table I). As was expected, a linear relationship between log k' and the carbon number of the MK side-chain was

TABLE I

CAPACITY RATIOS OF VITAMIN K COMPOUNDS IN ADSORPTION, REVERSED-PHASE AND CYANO-BONDED PHASE CHROMATOGRAPHY

Compound	*Adsorption, silica column*		*Reversed-phase*					*Cyano-bonded column, 0.05% acetonitrile in n-hexane eluent*
			C_{18} *HL column*			C_8 *column, 100% acetonitrile eluent*	C_{18} *LL column, 8% dichloromethane in acetonitrile eluent*	
	0.2% acetonitrile in n-hexane eluent	*10% dichloromethane in n-hexane eluent*	*100% methanol eluent*	*50% methanol in acetonitrile eluent*	*100% acetonitrile eluent*			
cis-K_1	4.4	5.8*	7.6	8.7	13.0	5.6	6.2	4.2
trans-K_1	5.2	6.9*						4.6
K_1 epoxide	6.1	6.5*	4.7	5.2	7.3	4.2	4.2	5.5
MK-2	8.7*	—**	1.8	1.7	1.9	1.7	2.1	—**
MK-4	8.8*	8.8	4.3	4.2	5.1	3.0	3.5	7.5
MK-9	9.5	10.3	—**	—**	—**	27.7	—**	9.0

* Not resolved from each other.

** Not determined.

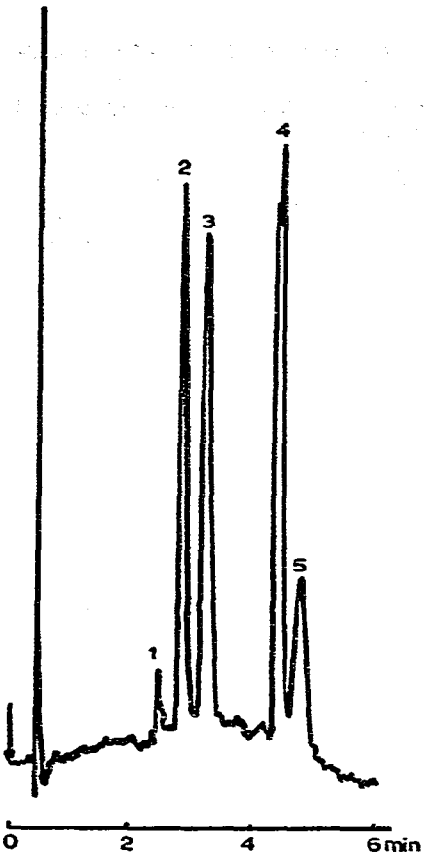


Fig. 1. Separation of vitamin K compounds by adsorption chromatography. Column: 15 cm × 3.2 mm I.D. silica (5 μm). Mobile phase: 0.2% acetonitrile in *n*-hexane, 1 ml/min. Peaks: 1 = *cis*-K_1; 2 = *trans*-K_1; 3 = K_1 epoxide; 4 = MK-2 + MK-4; 5 = MK-9.

observed[13]. Two important observations were made on all reversed-phase systems. Firstly, acetonitrile acts as a weaker eluent than methanol, in contrast to what one might expect on the basis of polarity. This was in close agreement with results obtained in our laboratory with other fat-soluble vitamins. Secondly, when acetonitrile was added to methanol, the selectivity and efficiency increased and the optimal solvent velocity shifted to higher values (Tables II and III). In particular, this increase in selectivity was important for the resolution of the pair MK-4–K_1 epoxide.

A high-load RP-18 HL 15 cm × 3.2 mm I.D. column separated K_1 well from interfering serum compounds still present after pre-purification on silica, but the sensitivity for K_1 was lower than in any other reversed-phase system, owing to the high capacity ratio (k'). The latter could be reduced by substituting C_8 for the high-load C_{18} packing. However, lower efficiencies for K_1 were obtained then and the maximal amount of serum that could be injected on to a 15 cm × 3.2 mm I.D. RP-8 column was 0.5 ml. Above this limit, the K_1 peak was no longer completely resolved from interfering compounds. Low-load C_{18} phase had the advantage of lower k' values than the high-load C_{18}, combined with a better resolution of K_1 from

TABLE II

INFLUENCE OF CONCENTRATION OF ACETONITRILE IN METHANOL IN REVERSED-PHASE CHROMATOGRAPHY OF VITAMIN K COMPOUNDS

Column, 15 cm × 3.2 mm I.D. RP-18 HL (10 μm); flow-rate 0.5 ml/min. N = number of theoretical plates; k' = capacity ratio; α = selectivity factor; R_S = resolution.

Acetonitrile in methanol (%)	*N for* K_1	*k'*			*α for* K_1 *epoxide–MK-4*	*R_S for* K_1 *epoxide–MK-4*
		K_1	K_1 *epoxide*	*MK-4*		
0	3400	7.6	4.7	4.3	1.09	1.0
25	3500	8.3	5.0	4.2	1.19	1.5
50	3600	8.7	5.2	4.2	1.24	2.2
100	5200	13.0	7.3	5.1	1.42	4.2

TABLE III

EFFECT OF PARTICLE DIAMETER AND NATURE OF ELUENT ON REVERSED-PHASE CHROMATOGRAPHY OF VITAMIN K_1

Columns: 15 cm × 3.2 mm I.D.

Parameter	*RP-18 LL (10 μm)*		*RP-18 LL (5 μm)*	
	100% methanol eluent	*100% acetonitrile eluent*	*100% acetonitrile eluent*	*8% dichloromethane in acetonitrile eluent*
Capacity ratio, k'	4.5	8.0	10.0	6.2
Optimal solvent velocity, u (cm/sec)	0.08	0.12	0.26	0.17
Number of theoretical plates, N	5200	6600	11,500	11,200
Reduced plate height, h	2.88	2.27	2.61	2.67

serum components than that obtained on C_8. Capacity ratios (k') and detection limits for K_1 are given in Tables I and IV. We packed 15 cm × 3.2 mm I.D. columns with both 10- and 5-μm RP-18 LL particles.

Although better reduced plate heights and lower k' values were obtained with the 10-μm packing, the 5-μm packing showed a significantly higher total plate number and therefore, increased sensitivity, as well as a higher optimal solvent

TABLE IV

DETECTION LIMITS FOR VITAMIN K_1 AT 248 nm ON 15 cm × 3.2 mm I.D. COLUMNS (SIGNAL TO NOISE RATIO = 3)

Parameter	*Phase*				
	RP-18 HL	*RP-8*	*RP-18 LL*	*RP-18 LL*	*RP-18 LL*
Particle size (μm)	10	5	10	5	5
Eluent	100% acetonitrile	100% acetonitrile	100% acetonitrile	100% acetonitrile	8% dichloro-methane in aceto-nitrile
N	6500	3300	6600	11,500	11,200
k'	13.0	5.6	8.0	10.0	6.2
Detection limit	4 ng	1.3 ng	1.8 ng	900 pg	550 pg

velocity (Tables III and IV). For practical work, higher solvent velocities (0.54 cm/sec, corresponding to a flow-rate of 1 ml/min) could be used, without affecting the separation, to reduce the total analysis time. The capacity ratio was adjusted by adding dichloromethane (up to 8%) to acetonitrile in order to obtain optimal sensitivity. The same result could also be obtained by using tetrahydrofuran–acetonitrile mixtures. With 100% methanol, k' values in the same range were obtained, but the column efficiencies were lower. The separation of MK-2, MK-4, K_1 epoxide and K_1 on RP-18 LL (5 μm) is shown in Fig. 2. Reversed-phase chromatography on smaller columns (10 cm × 2 mm I.D.) has been investigated but abandoned as the increase in absolute sensitivity[23] was counteracted by the very low sample capacity. We also found it difficult to pack these columns reproducibly.

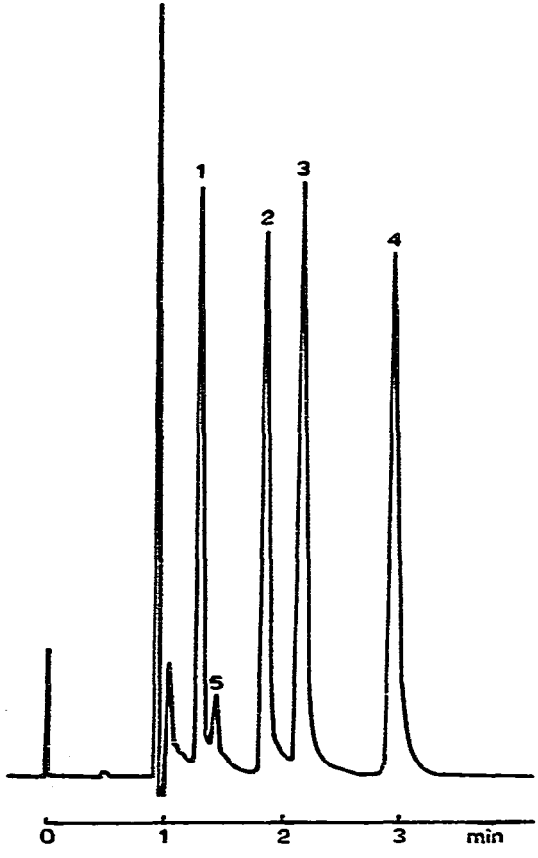


Fig. 2. Separation of vitamin K compounds by reversed-phase chromatography. Column: 15 cm × 3.2 mm I.D. RP-18 LL (5 μm). Mobile phase: 8% dichloromethane in acetonitrile, 1 ml/min. Peaks: 1 = MK-2; 2 = MK-4; 3 = K_1 epoxide; 4 = K_1; 5 = unknown impurity.

A third chromatographic system on cyano (CN) bonded phase was developed later to confirm the results obtained from serum extracts with the combined adsorption–reversed-phase method. We tried the CN column in both the reversed- and the straight-phase modes. While no retention for vitamin K analogues was obtained in

the reversed-phase mode, a similar pattern to that for adsorption chromatography was observed when we eluted the column with 0.05% acetonitrile in *n*-hexane (Fig. 3). Again, the superiority of acetonitrile over dichloromethane was illustrated as we obtained only two peaks with dichloromethane–*n*-hexane mixtures: one for K_1 (*cis* + *trans*) and K_1 epoxide and a second for the menaquinones. To obtain k' values of the same order of magnitude as those found on a silica column, the eluotropic strength (ε_0) has to be lower on the CN-phase for all vitamin K compounds (Table I).

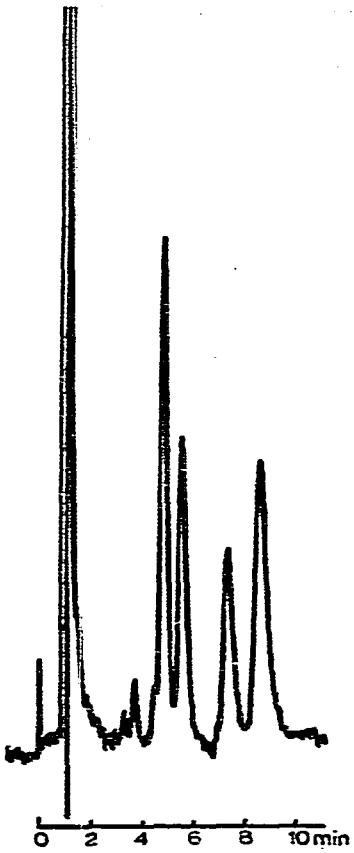


Fig. 3. Separation of vitamin K compounds by chromatography on cyano-bonded phase. Column: 15 cm × 3.2 mm I.D. Rsil CN (5 μm). Mobile phase: 0.05% acetonitrile in *n*-hexane, 0.5 ml/min. Peaks: *cis*- + *trans*-K_1 (4.8 min); K_1 epoxide (5.6 min); MK-4 (7.4 min) and MK-9 (8.6 min).

As overlapping serum peaks hindered the quantitation of K_1 on both silica and reversed-phase columns, we chose a two-column approach. The serum extracts were pre-purified and fractionated on a 20 cm × 4.6 mm I.D. silica column. Amounts corresponding to up to 2 ml of serum could be injected. The eluent was monitored at 254 nm and the portions of the eluent containing the compounds of interest were identified after injection of an extract, spiked with K_1, K_1 epoxide and MK-4. The three fractions collected for the determination of K_1, K_1 epoxide and MK-4 are

indicated by arrows in Fig. 4. We focused our attention mainly on the determination of vitamin K_1, especially as a suitable internal standard was available. When a shorter column (10 cm × 4.6 mm I.D.) was used, or larger collection windows were applied, considerably more contamination in the reversed-phase chromatograms occurred.

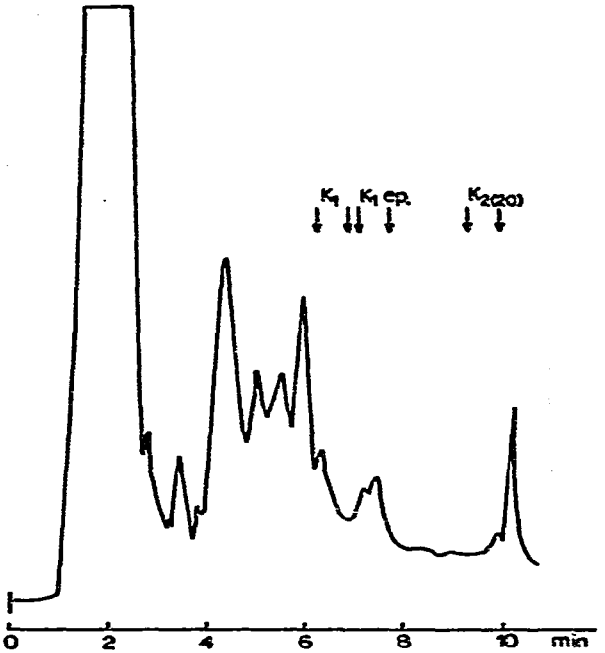


Fig. 4. Pre-purification of an extract of 1.5 ml of serum. Column: 20 cm × 4.6 mm I.D. silica (5 μm). Eluent: 0.2% acetonitrile in *n*-hexane, 1.5 ml/min. Fractions containing K_1, K_1 epoxide and MK-4 are indicated.

The sample capacity of 3.2 mm I.D. silica columns was insufficient. The use of a short guard column (5 cm × 4.6 mm I.D. Perisorb A, 30–40 μm) is recommended for protecting the analytical silica column. Injections could be made every 20 min when the system was operated at a flow-rate of 2 ml/min. The chromatogram in Fig. 5 was obtained by injecting a portion of the silica K_1 eluate on to a 15 cm × 3.2 mm I.D. C_{18} LL (5 μm) column. Amounts corresponding to up to 1 ml of serum could be injected without problems. This brings the minimal detectable amount of vitamin K_1 in serum down to about 500 pg/ml (Table IV). We chromatographed serum extracts at both 270 nm (quinoid absorption band of K_1) and 248 nm (benzenoid absorption band) but no marked increase in selectivity was observed at 270 nm, while the sensitivity is better at 248 nm.

We used *trans*-[^{3}H]-K_1 as internal standard as it accounts not only for losses during the extraction and evaporation steps, but also for the accuracy of the collection of the K_1 fraction on silica. The fraction of reversed-phase eluent containing the K_1 peak was collected for scintillation counting of the internal standard (Fig. 5). The concentration of K_1 in serum was determined from the peak height to dpm ratio.

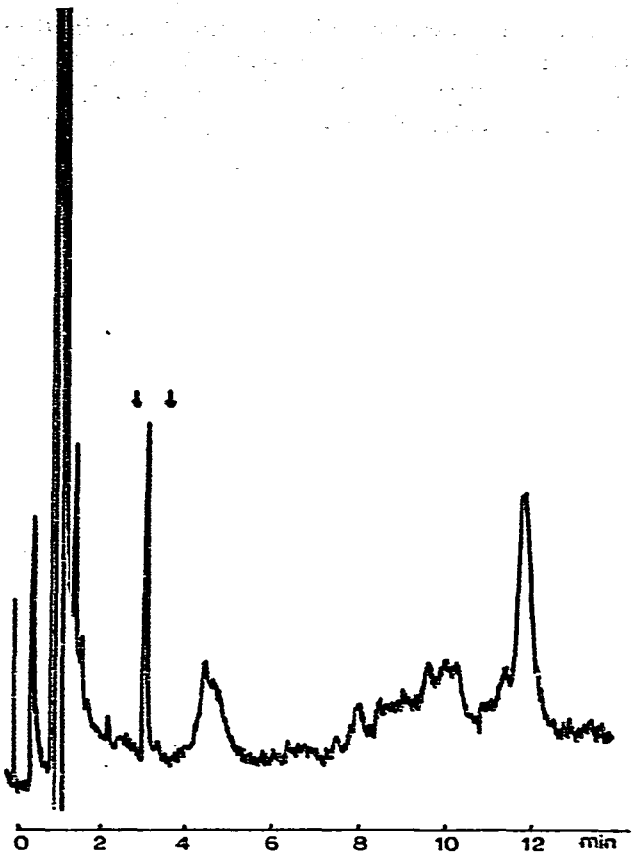


Fig. 5. Chromatography on RP-18 LL of the K_1 fraction obtained after pre-purification on silica. The injected amount corresponds to 0.5 ml of serum extracted. The arrows indicate the K_1 peak, corresponding to 4 ng. Column: 15 cm × 3.2 mm I.D. RP-18 LL (5 μm). Mobile phase: 8% dichloromethane in acetonitrile, 1 ml/min. Detection: UV, 248 nm, 0.005 a.u.f.s.

Extraction recoveries on basis of the number of dpm of *trans*-[^{3}H]-K_1 extracted from serum averaged 93.9 ± 2.2% (coefficient of variation = 2.4%, $n = 7$).

The total recovery of the method was determined by the ratio of the specific activity of the K_1 peak in the final reversed-phase step to the activity originally added to serum. Values from 60 to 89% were found.

The identity of the K_1 peak was confirmed by the UV absorption spectrum obtained by injecting equal amounts of serum extract at different wavelength settings. Maxima were observed at 243, 248, 270 and 320 nm, the quinoid maximum at 262 nm being less pronounced. The same spectrum was obtained from a standard solution of vitamin K_1 and correlates closely with data given in the literature[24]. On both silica and nitrile 15 cm × 3.2 mm I.D. columns, a peak with k' equal to that of vitamin K_1 was observed upon injection of the K_1 peak collected from the reversed-phase column (Fig. 6). The results of the spectrometry and the re-chromatography together with the specificity of the two-column system not only confirm the identity of the vitamin K_1 peak, but also suggest that the presence of another peak with k' equal to that of

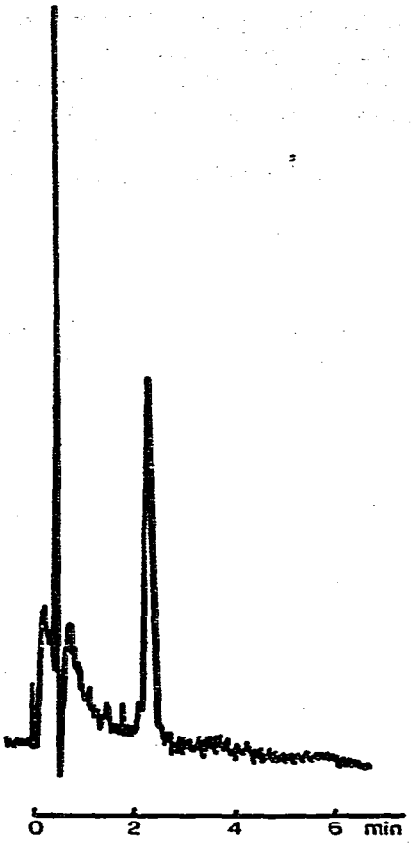


Fig. 6. K_1 peak (11 ng) collected from C_{18} LL (5 μm) on cyano-bonded phase. Column: 15 cm × 3.2 mm I.D. Rsil CN (10 μm). Eluent: 0.05% acetonitrile in *n*-hexane, 1 ml/min. Detection: UV, 248 nm, 0.005 a.u.f.s.

K_1 in the reversed-phase chromatogram, which absorbs UV radiation at 248 nm, is very improbable.

Vitamin K_1 serum levels in our studies to date on normal healthy adults range between 5 and 30 ng/ml. In patients we found a marked increase in the vitamin K_1 level of serum (70–80 ng/ml) 1 h after the administration of a dose of 10 mg of K_1.

CONCLUSION

In the separation of vitamin K analogues by adsorption, reversed-phase and cyano-bonded phase chromatography, the sensitive detection of vitamin K_1 was possible by using a low-loading octadecyl phase and a modern variable-wavelength detector. Levels down to 500 pg/ml in serum could be detected after fractionation of the serum extracts on silica.

The use of a two-column system in combination with detection of the compound of interest at its $\lambda_{max.}$ provides high selectivity. The results of UV absorption

spectrophotometry and re-chromatography confirmed the identity of the vitamin K_1 peak obtained from serum and suggested that no overlapping peaks were present.

Tritiated vitamin K_1 is a good internal standard in this instance as it compensates both for variations in the extraction and evaporation steps and for losses during the collection of the vitamin K_1 fraction on silica.

Serum levels (5–30 ng/ml) found under physiological conditions are of the order of 10 times above the detection limit of the method.

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