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RESOLUTION OF MENAQUINONES (VITAMINS K_2) BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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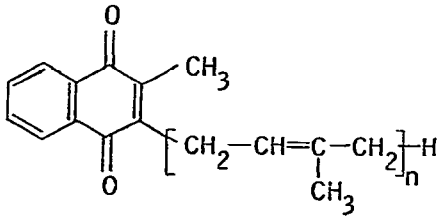
SUMMARY

The separation of menaquinones (MKs) and phylloquinone (K_1) by high-performance liquid chromatography (HPLC) was investigated using silica, cyano-bonded and reversed-phase supports. On silica and cyano-bonded supports it was possible to completely resolve *cis*- and *trans*-K_1, MK-4 and MK-10, though MKs with intermediate side chain lengths were incompletely resolved from each other. Partially saturated MKs with the same side chain length could be completely separated from each other or from their parent quinones on a column of Spherisorb-5 silica when the difference between them was equal to or greater than two saturated prenyl units.

A mixture of K_1 and MKs 4–10 which included the saturated forms of MK-8(H_4), MK-9(H_6) and MK-9(H_8) could be resolved by reversed-phase HPLC within 30 min.

INTRODUCTION

Menaquinones (MKs) are found exclusively in bacteria. Since the isolation of both menaquinone-6 (MK-6) and menaquinone-7 (MK-7) from putrefied fish meal by Isler *et al.*[1], each successive member of the series from MK-6 to MK-13 has been isolated either from specific bacterial cultures or from mixed bacterial populations. In addition several modified menaquinones have been isolated from bacteria in which one or more of the double bonds of the prenyl units have been reduced (partially saturated menaquinones).



Menaquinones (MK-n)

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Knowledge about the content and types of K vitamins found in animal tissues has been made possible by developments in chromatographic techniques whereby the several molecular forms could be resolved, both from each other and from contaminating lipids. For example, the discovery of high-molecular-weight menaquinones in beef liver[2,3] was made possible by the development of reversed-phase columns[2,4] and thin-layer techniques[3,5], which were based on the partition coefficient of K vitamins between immiscible solvents such as liquid paraffin and aqueous acetone. Although these methods have proved useful for the separation of the major forms of menaquinones which differ in the number of isoprene units in the side chain[6] they do not completely resolve those menaquinones with the same length of side chain but with varying degrees of saturation of the double bonds. To resolve these partially saturated menaquinones Matschiner and Amelotti[3] employed argentation chromatography. Adsorbents impregnated with silver ions are capable of resolving menaquinones on the basis of the number of double bonds present in the isoprenoid side chain. Although this technique has been of value for the tentative identification of partially saturated forms of menaquinones from animals[5,7] and microbial[8] sources, its use is limited because of irreversible adsorption of high-molecular-weight menaquinones to the silver nitrate impregnated silica. In view of these findings, the development of high-performance liquid chromatographic (HPLC) methods for the separation of K vitamins is of obvious value.

Besides the applicability of HPLC methods for the resolution of K vitamins from animal sources we also considered that HPLC would offer a superior tool to the microbiologists interested in the study of menaquinones synthesized by bacteria[8–12].

Previously, bacterial menaquinones have been isolated by reversed-phase paper[9–12], thin-layer[8,13] or argentation chromatography[8]. These techniques are time consuming and in some instances require chromatography for up to 20 h to obtain pure preparations of menaquinones[11].

In order to overcome at least some of the limitations of conventional methods used in the analyses of K vitamins we have developed HPLC methods for the efficient resolution of these compounds which we consider will provide a valuable aid for future studies of the biochemistry of menaquinones. Although the separation of menaquinones by reversed-phase HPLC has been reported previously, these have been limited mainly to low-molecular-weight forms[14,15]. Recently we have reported on the resolution of phylloquinone and related compounds by HPLC[16] and also briefly on the separation of menaquinones 4–10 (ref. 17). In this communication we describe in detail the chromatography of menaquinones including partially saturated forms of these compounds by both adsorption and reversed-phase modes of HPLC.

EXPERIMENTAL

Chemicals

Vitamins K_1 (phylloquinone) and K_2 (MKs 4–10) were gifts from Hoffmann-La Roche (Basle, Switzerland). Phylloquinone 2,3-epoxide (K_1 epoxide) was synthesized as described previously[16]. Two other preparations of partially-saturated menaquinones were obtained from Dr. Y. Yamada, Shizuoka University, Shizuoka, Japan, and used without further purification. One preparation mainly consisted of MK-8(H_4) and was isolated from the genus *Nocardia*. The second preparation mostly consisted of

MK-9(H_6) and -9(H_8) and these were isolated from the genus *Streptomyces*. HPLC grade solvents were purchased from Rathburn (Walkerburn, Great Britain), and 50% water-saturated dichloromethane was prepared as described previously[16].

HPLC

Details of HPLC assembled using commercially available components have already been published[16]. In addition, we employed in these studies a gradient elution programmer (Model 750/30) obtained from Applied Chromatography Systems (Luton, Great Britain). The solvent gradients developed to resolve menaquinones from a Zorbax-ODS (250 × 4.6 mm I.D., DuPont, Hitchin, Great Britain) column were as follows. In system 1 the concentration of dichloromethane in methanol was increased at a rate of 5%/min from an initial concentration of 20% to a final concentration of 50%. In system 2 the rate (5%/min) of addition of dichloromethane to acetonitrile was increased from an initial concentration of 20% to a final concentration of 60%. In system 3 the rate (10%/min) of addition of tetrahydrofuran to acetonitrile was increased from an initial concentration of 20% to a final concentration of 40%. These gradients were run at constant pressure (40 bar) with an initial flow-rate of 1 ml/min. For isocratic elution at ambient temperature the flow-rate was 1 ml/min. For adsorption HPLC the following columns were employed: Spherisorb-5 and Spherisorb-5CN were supplied by Phase Separations (Queensferry, Great Britain), and packed in columns (250 × 5 mm I.D.) by HPLC Technology (Wilmslow, Great Britain), and Hichrom (Woodley, Great Britain), respectively. Partisil-10PAC was obtained pre-packed in a column (250 × 4.6 mm I.D.) from Whatman (Maidstone, Great Britain), and the Partisil-5 was supplied by Whatman and was packed into columns (250 × 4.9 mm I.D.) by Hichrom.

For reversed-phase HPLC samples were dissolved in the initial mobile phase and injected with a syringe and on-column septum injector (HPLC Technology). In the case of adsorption HPLC, samples were dissolved in hexane and injected as described previously[16].

RESULTS

Adsorption and cyano-bonded HPLC

Table I shows the capacity ratios of K vitamins on systems of adsorption and cyano-bonded HPLC. With dichloromethane as a moderator in hexane, both types of microparticulate supports whilst completely resolving MK-4 from MK-10 only partially separated isoprenologues with intermediate side-chain lengths. In addition, these supports could also completely separate MKs 4–10 from *cis*- and *trans*-phylloquinone (Table I). Although the activity of silica and cyano-bonded supports may be controlled by employing dry dichloromethane as a moderator in hexane, this resulted in considerable peak tailing and low column efficiencies. To circumvent these problems we deactivated the silica by using mixtures of 50% water-saturated dichloromethane in hexane. As illustrated by the chromatogram in Fig. 1, a column of Spherisorb-5 with a mobile phase of water-saturated dichloromethane in hexane gave higher column efficiencies and decreased peak asymmetry for K vitamins.

No difference in the resolution of K vitamins was found on silica supports when the polarity of the moderator was increased by replacing dichloromethane with

TABLE I

CAPACITY RATIOS OF VITAMIN K COMPOUNDS BY ADSORPTION AND CYANO-BONDED HPLC

Compound	*Mobile phase, column and flow-rate*					
	25% Dichloromethane (50% water-saturated) in n-hexane		*0.5% Acetonitrile in n-hexane*		*20% Dichloromethane (50% water-saturated) in n-hexane*	*10% Dichloromethane (50% water-saturated) in n-hexane*
	Partisil-5, 2.0 ml/min	*Spherisorb-5, 1.0 ml/min*	*Partisil-5, 1.0 ml/min*	*Spherisorb-5, 1.0 ml/min*	*Partisil-10PAC, 1.0 ml/min*	*Spherisorb-5CN, 1.0 ml/min*
cis-K_1	2.8	1.3	1.1	0.9	0.8	1.2
trans-K_1	3.4	1.7	1.2	1.1	1.0	1.4
MK-10	5.1	2.2	1.5	1.3	1.2	1.7
MK-4	6.2	2.7	1.8	1.5	1.5	2.0

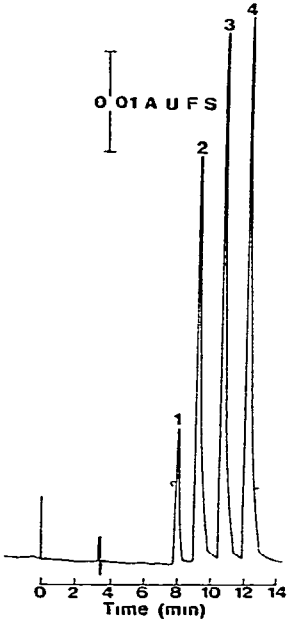
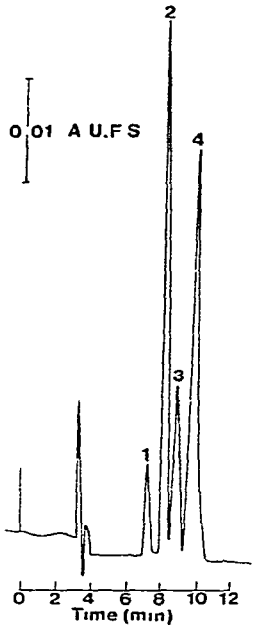


Fig. 1. Separation of vitamin K compounds by adsorption HPLC on Spherisorb-5 with a mobile phase of 25% dichloromethane (50% water-saturated) in hexane and a flow-rate of 1 ml/min. Detection was at 250 nm. Peaks: 1 = *cis*-K_1; 2 = *trans*-K_1; 3 = MK-10; 4 = MK-4.

acetonitrile. For example, silica packings of irregular (Partisil-5) and spherical shape (Spherisorb-5) could still resolve MK-4 from MK-10 and also these from *cis*- and *trans*-K_1 (Table I). In addition, the degree of selectivity between MK-4 and MK-10 on the acetonitrile based system was found to be similar to the dichloromethane based system. These mobile phases also had no effect on the order of elution of K vitamins (Table I).

The resolution of partially-saturated menaquinones of bacterial origin was also examined on a silica (Spherisorb-5) column. With a mobile phase of dichloromethane in hexane, hydrogenated forms of MK-8 and -9 were retained to a lesser extent than the parent quinone and could be resolved from them (Figs. 2 and 3). Although MK-8(H_4) was retained longer on Spherisorb-5 than either MK-9(H_6) or -9(H_8) it could only be partially resolved from these forms of the higher isoprenologue (Figs. 2 and 3). An interesting finding during the adsorption chromatography of MK-8(H_4), MK-9(H_6) and MK-9(H_8) was that each individual member could be resolved into a minor and major component, as was *cis*- and *trans*-K_1 (Figs. 2 and 3). On reversed-phase HPLC, the two components eluted as a single peak and were chromatographically distinct from MK-8, -9 and -10. These chromatographic properties suggested that the major and minor components differed from each other, not in their side-chain length or degree of saturation, but rather by a geometric factor. It is possible that the minor component is the *cis*-isomer of the saturated forms of MK-8 and -9. These have been previously reported by Dunphy *et al.*[13] from *Mycobacterium phlei* and also by Yamada *et al.*[11] from *Oerskovia turbata* and *Brevibacterium lipolyticum* in the nocardioform and coryneform groups of bacteria



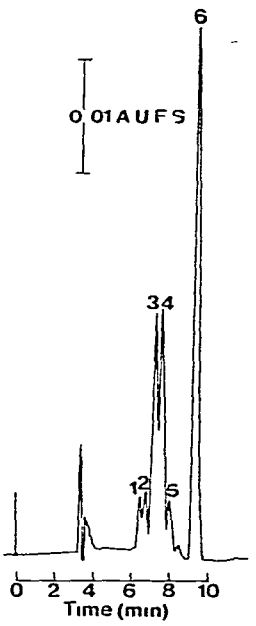


Fig. 2. Separation of MK-8 and MK-8 (H_4) by adsorption HPLC on Spherisorb-5 with a mobile phase of 25% dichloromethane (50% water-saturated) in hexane and a flow-rate of 1.5 ml/min. Detection was at 250 nm. Peaks: 1 = MK-8 (H_4) minor; 2 = MK-8 (H_4) major; 3 = unknown; 4 = MK-8.

Fig. 3. Separation of MK-9, MK-9 (H_6) and MK-9 (H_8) by adsorption HPLC on Spherisorb-5 with a mobile phase of 25% dichloromethane (50% water-saturated) in hexane and a flow-rate of 1.5 ml/min. Detection was at 250 nm. Peaks: 1 = MK-9 (H_8) minor; 2 = MK-9 (H_6) minor; 3 = MK-9 (H_8) major; 4 = MK-9 (H_6) major; 5 = unknown; 6 = MK-9.

respectively. The ratio of the minor to the major components of MK-8(H_4) (isolated from a nocardioform bacteria) determined by HPLC was found to be 1:6. This ratio is similar to those ratios for MK-9(H_6) and -9(H_8) (isolated from *Streptomyces*) which was found to be approximately 1:5 respectively.

Reversed-phase HPLC

The separation of menaquinones under both isocratic and gradient elution was examined on a commercially available ODS phase bonded to silica (Zorbax-ODS).

Isocratic elution. Although with mixtures of dichloromethane in methanol it was possible to resolve completely MKs 4–10, K_1 and K_1 epoxide, the separation between MK-6 and K_1 was lost or at best was partial when methanol was replaced by acetonitrile (Table II). The linear relationship between the log of capacity factors for MKs 4–10 and the carbon number of the side-chain was observed with solvent pairs based on methanol and acetonitrile[18]. These findings are in agreement with the observation of other workers[14,15] who found the same linear relationship for a more limited number of menaquinones. The capacity ratios of MK-8(H_4), -9(H_6) and -9(H_8) on a methanol based system are shown in Table II. Saturation of the double

TABLE II

CAPACITY RATIOS OF K VITAMINS BY REVERSED-PHASE HPLC AND ISOCRATIC ELUTION ON ZORBAX-ODS

— Not determined.

Compound	*Mobile phase*	
	30% Dichloromethane in acetonitrile	*30% Dichloromethane in methanol*
MK-4	0.9	0.9
MK-5	1.4	1.3
K_1	2.1*	1.5
MK-6	2.1*	1.8
MK-7	3.1	2.4
MK-8	4.6	3.3
MK-9	6.8	4.4
MK-8 (H_4)	—	4.9
MK-10	9.8	5.8
MK-9 (H_6)	—	6.5
MK-9 (H_8)	—	7.6

* Denotes compounds not resolved from each other.

bonds in the isoprene units resulted in increased retention and these menaquinones behaved like higher isoprenologues[8,9]. Thus partially-saturated MK-8 and -9 could be completely resolved from their parent quinones (Table II). In contrast to adsorption HPLC, menaquinones differing by a single double bond [*e.g.* MK-9(H_6) and MK-9(H_8)] could be completely resolved on Zorbax-ODS with mixtures of dichloromethane in methanol (Table II). In addition, the separation between these and MK-8(H_4) was also achieved by reversed-phase HPLC (Table II). The order of elution of MK-9(H_6) before MK-9(H_8) was evaluated by consideration of the pattern of retention for the pair of compounds, K_1 and MK-4. Since K_1 eluted after MK-4 and K_1 may be considered as a saturated form of MK-4, more specifically MK-4(H_6), the data suggested that the isoprenologue with the greatest degree of saturation would elute last.

Gradient elution. Fig. 4 shows a typical resolution of K vitamins and K_1 epoxide achieved on Zorbax-ODS employing a non-aqueous mobile phase of dichloromethane in methanol. Gradient elution resulted in a shorter and more convenient resolution of K vitamins and it was possible to obtain base-line separation of eleven different K vitamins in a single run within 30 min. Although a similar separation of MKs 4–10 could also be achieved with mixtures of dichloromethane or tetrahydrofuran in acetonitrile these solvent pairs could not resolve MK-6 from K_1 (Table III).

DISCUSSION

The primary goal of this study was to develop rapid methods of HPLC for the separation of menaquinones. The advantages of these HPLC systems include high resolution and greater sensitivity.

Straight and cyano-bonded HPLC systems reported here were all able to resolve the highly lipophilic MK-10 from the less lipophilic MK-4. In contrast to our

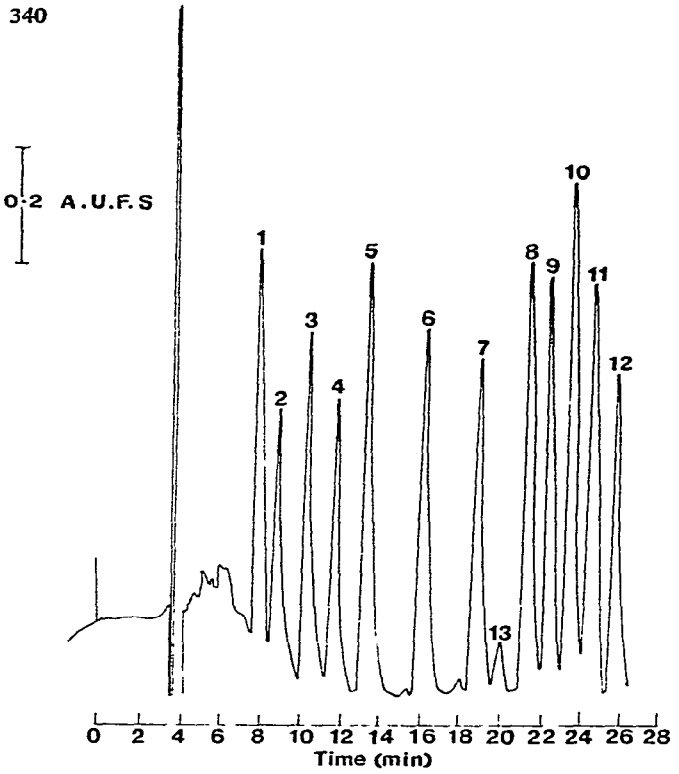


Fig. 4. Separation of vitamin K compounds by reversed-phase HPLC on Zorbax-ODS using gradient elution, System 1 (see Experimental). Detection was at 250 nm. Peaks: 1 = MK-4; 2 = K_1 epoxide; 3 = MK-5; 4 = K_1; 5–8 = MK-6–MK-9; 9 = MK-8 (H_4); 10 = MK-10; 11 and 12 = MK-9 (H_6) and MK-9 (H_8); 13 = unknown.

TABLE III

RETENTION TIMES* (min) OF K VITAMINS BY REVERSED-PHASE HPLC AND GRADIENT ELUTION ON ZORBAX-ODS

— Not determined.

Compound	*Mobile phase and gradient elution*		
	Dichloromethane in methanol (Initial 20%, final 50%)	*Dichloromethane in acetonitrile (Initial 20%, final 60%)*	*Tetrahydrofuran in acetonitrile (Initial 20%, final 40%)*
MK-4	8.1	6.0	7.0
MK-5	10.5	8.0	9.1
K_1	11.8	10.2**	10.9**
MK-6	13.4	10.2**	10.9**
MK-7	16.2	12.6	12.9
MK-8	19.0	14.8	14.5
MK-9	21.4	16.7	15.8
MK-8 (H_4)	22.4	—	—
MK-10	23.5	18.4	17.5
MK-9 (H_6)	24.6	—	—
MK-9 (H_8)	25.8	—	—

* Values are expressed as retention times because the flow-rate was not constant during gradient elution.

** Denotes compounds not resolved.

results showing that MK-10 eluted before MK-4 from both Partisil and Spherisorb silicas, Lefevere *et al.*[15] reported that on a silica packing (Rsil, 5 μm) using acetonitrile as moderator in hexane, low-molecular-weight menaquinones (MK-1 and MK-4) eluted before MK-9. Our data (Table I) indicate that the resolution of menaquinones on silica and cyano-bonded supports is related to their polarity and that at least on silica neither the shape of the column packing particles (spherical or irregular) nor the types of moderator (dichloromethane or acetonitrile) had any effect on the order of elution of menaquinones. This is supported by our findings that partially-saturated forms of MK-8 and -9 which were more lipophilic than the parent quinones were retained on silica to a lesser extent than MK-8 and -9.

Besides being able to resolve MKs 4–10, the reversed-phase system elucidated here was also capable of separating partially saturated forms of the same or different isoprenologues. In particular, reversed-phase HPLC gave a better resolution of partially saturated forms of menaquinones differing by a single saturated double bond [*i.e.* MK-9(H_6) and MK-9(H_8)] than previously possible by reversed-phase thin-layer chromatography[19]. Since these forms of K vitamins occur in nature the system we have developed offers a more sensitive and rapid method of separating these compounds from both animals and microbial sources. In this respect, there is evidence to suggest that coryneform and nocardioform micro-organisms may be further classified taxonomically on the basis of the menaquinones they synthesize[9–12]. In our view HPLC would confer many advantages to these taxonomic studies which have previously been carried out using reversed-phase paper chromatography. In addition, the application of these HPLC methods could also facilitate previously prohibitive and tedious analyses of K vitamins from animal sources.

In a previous communication we reported on the advantages of non-aqueous over semi-aqueous mobile phases in the analyses of phylloquinone and related compounds by reversed-phase HPLC[16]. These advantages may now be extended to the analyses of menaquinones especially the highly lipophilic hydrogenated forms. Their increased solubility in the non-aqueous carrier liquids means that high sample loads can be applied to columns wihch in turn aids their detection, especially from animal sources where they are present in low concentrations.

The non-aqueous mobile phases examined offered different selectivities. For example, although K_1 and MKs 4–10 could be resolved with mixtures of dichloromethane in methanol it was not possible to separate K_1 and MK-6 with mixtures of dichloromethane or tetrahydrofuran in acetonitrile. Against this, acetonitrile-based systems gave higher selectivity over methanol-based systems for the resolution of K_1 and chloro-K_1 (ref. 16). Although gradient and isocratic elution produced similar chromatography of K vitamins on reversed-phase HPLC, the advantage of gradient elution was that the technique offered a more rapid and efficient separation of menaquinones.

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