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Note

High-performance liquid chromatography of menaquinone-4, 2,3-epoxymenaquinone-4, demethylmenaquinone-4 and related compounds

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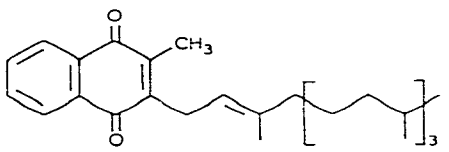
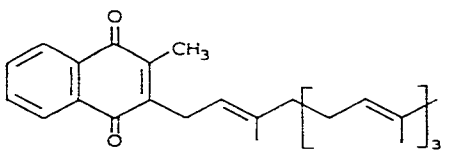
It has been shown recently that prothrombin and other blood clotting proteins contain γ-carboxy glutamate residues which confer calcium binding properties to these proteins[1]. Vitamin K is closely implicated in the carboxylation of glutamate residues in precursor proteins to form these blood clotting proteins[2]. γ-Carboxyglutamate residues are also found in other calcium binding proteins and presumably vitamin K is required for their synthesis[3]. Vitamin K is a term which encompasses several methyl naphthoquinone derivatives and the most biologically important compounds are phylloquinone (I) a plant product and menaquinones (II) formed by bacteria. In spite of



Phylloquinone

I



Menaquinone-4

II

the vitamin status, both vertebrates and invertebrates can form menaquinone-4 (MK-4) when supplied with menadione (2-methyl-1:4-naphthoquinone)[4–6]. 2,3-Epoxy derivatives of these compounds appear to be important. When phylloquinone is administered to rats 2,3-epoxyphylloquinone can be detected in the liver[7] and 2,3-epoxy MK-4 is formed in invertebrates together with MK-4 when menadione is given[6]. Another derivative found experimentally is 2-demethyl MK-4 which is produced in invertebrates when given 1:4-naphthoquinone[8] and demethyl menaquinones are implicated generally in MK biosynthesis in bacteria[9]. Thus studies on the biosynthesis or function of vitamin K may require the separation of several derivatives

and homologues. The thin-layer chromatographic (TLC) properties of phylloquinone and various menaquinone isoprenologues were collated by Sommer and Kofler[10]. Adsorption, reversed-phase and argentation systems were described but neither 2,3-epoxyderivatives nor 2-demethyl derivatives were examined. Separation of a 2,3-epoxy derivative of MK-4 from the parent compound has been described[6] but of the three chromatographic methods described above, only reversed-phase TLC was successful. On adsorption TLC 2-demethyl menaquinones have similar R_F values to the parent menaquinones[11]. The present paper describes R_F values for menaquinones and some derivatives on three TLC systems and it is clear that complete separation of vitamins K cannot be achieved on any one system. Therefore the development of a high-performance liquid chromatographic (HPLC) method to separate these compounds in one step is of great value.

EXPERIMENTAL

Chemicals

Phylloquinone was obtained from Koch-Light Laboratories (Colnbrook, Great Britain) and menadione was obtained from Sigma (St. Louis, Mo., U.S.A.). MK-3 and MK-4 were synthesized by condensing menadiol with nerolidol or geranyl-linalool respectively[12]. Epoxymenaquinones and epoxyphylloquinone were formed by the action of H_2O_2 in aqueous ethanol containing Na_2CO_3 on the relevant menaquinone[13]. 2-Demethyl MK-4 was formed by condensing 1:4-naphthoquinol with geranyl-linalool[12].

Thin-layer chromatography

Three systems were used: adsorption, argentation and reversed-phase TLC. In the first case the adsorbent was silica gel G and the solvent was 6% diethyl ether in light petroleum (b.p. 40–60°). Ag^+-Impregnated plates were prepared using 10% (w/w) $AgNO_3$–silica gel G and 60% diisopropyl ether in light petroleum as solvent. Diisopropyl ether normally contains hydroquinone to prevent peroxide formation and this was removed by passing the solvent through acid-washed alumina (Brockmann grade 0). Reversed-phase chromatography was carried out on paraffin-impregnated Kieselguhr G with 90% aqueous acetone as solvent. A 5% solution of liquid paraffin in light petroleum was passed down an acid-washed alumina column (Brockmann grade 0) to remove congeners from the paraffin which interfere with the separation and then Kieselguhr G plates were dipped in the solution and the light petroleum was allowed to evaporate. The aqueous acetone solvent was saturated with paraffin before use. All plates were 0.25 mm thick.

High-performance liquid chromatography

A Model ALC/100 liquid chromatograph (Waters Assoc., Northwich, Great Britain) fitted with a 254-nm UV monitor was used. Three systems were employed. (A) 3 × 30 cm μBondapak C_{18} column (particle size 10 μm) with acetonitrile–water (85:15 v/v) as solvent; (B) 2 × 30 cm μBondapak C_{18} column with methanol as solvent and (C) 2 × 25 cm Partisil 10 ODS (particle size 7 μm) with acetonitrile–water (66:34 v/v). The Bondapak C_{18} was obtained from Waters Assoc. and Partisil from Whatman, Maidstone, Great Britain). Other details are as described by Rees *et al.*[14].

RESULTS

The separation of MK-4 and its related derivatives (phylloquinone, 2,3-epoxide and 2-demethyl) were examined by TLC and HPLC. MK-3 and MK-3 oxide were also included since biosynthetic studies in invertebrates showed that both farnesol (C_{15}) and geranylgeraniol (C_{20}) were formed from mevalonic acid[14] and so synthesis of MK-3 and MK-4 in the presence of menadione seemed not unlikely.

Table I shows the results of TLC separations. On the adsorption and argentation systems phylloquinone and the synthetic menaquinones separated into two components corresponding with the 2',3'-*cis–trans* isomers of these compounds. The *cis* isomer usually amounting to about 10% of the total migrated just in front of (*i.e.* was less polar than) the *trans* isomer. All values given in Table I are for the *trans* isomer. The isomers were not detected in the epoxy derivatives or on reversed phase.

TABLE I

TLC OF VITAMIN K AND SOME DERIVATIVES

Compound	*TLC system*		
	*Adsorption** (R_F)	*Reversed-phase*** (R_F)	*Silver nitrate**** (R_F)
Menaquinone-4	0.29	0.66	0.48
2,3-Epoxymenaquinone-4	0.27	0.81	0.46
Demethylmenaquinone-4	0.27	0.72	0.45
Menaquinone-3	0.28	0.79	0.67
2,3-Epoxymenaquinone-3	0.27	0.89	0.59
Phylloquinone	0.35	0.38	0.97
2,3-Epoxyphylloquinone	0.33	0.59	0.91
Menadione	0.12	0.96	0.62

* Silica gel G–6% (v/v) diethyl ether in light petroleum.
** Paraffin-impregnated Kieselguhr–90% aq. acetone (v/v).
*** 10% (w/w) AgNO–silica gel G–60% (v/v) diisopropyl ether in light petroleum.

On the adsorption system all the derivatives migrated very similarly to one another. Although for example MK-4 and epoxy MK-4 had different R_F values it was not possible to separate a mixture of the two. On the reversed phase system each epoxy derivative moved well ahead of (was more polar than) the related quinone. However, MK-3 migrated in an almost identical manner to epoxy MK-4 on reversed phase and thus the two could easily be confused. On argentation TLC phylloquinone, MK-3 and MK-4 were readily separable but the 2,3-epoxy derivatives migrated in each case very close to their parent compounds. Demethyl MK-4 migrated in a similar manner to epoxy MK-4 on adsorption and argentation TLC but could be separated on reversed-chromatograms. Therefore to separate a mixture of MK-3, MK-4 their epoxy derivatives and demethyl MK-4 would require TLC on all three systems, *i.e.* adsorption, followed by reversed phase and finally argentation.

The initial fractionations by HPLC were carried out on μBondapak C_{18} columns. It was apparent that separations were excellent and in particular that it was necessary to use different eluting solvents for phylloquinone and the epoxy derivative on the one hand and MK-4, MK-3 and their epoxyderivatives on the other. In most

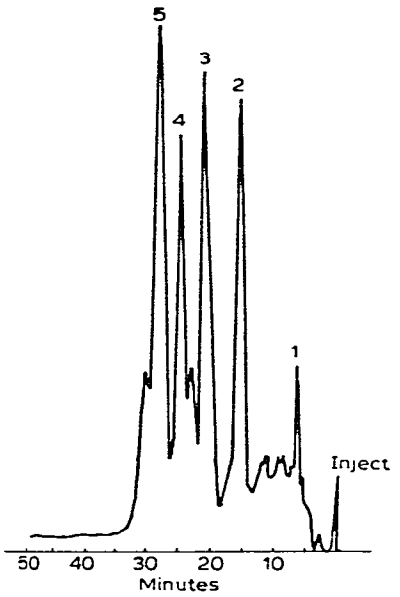


Fig. 1. Separation of some menaquinones using system C, 2 × 25 cm Partisil 10 ODS with acetonitrile–water (66:33, v/v). Peaks: 1 = menadione; 2 = menaquinone-3; 3 = 2,3-epoxymenaquinone-4; 4 = 2-demethylmenaquinone-4; 5 = menaquinone-4.

experiments only one system would be required as studies usually involve either phylloquinone or MK-4 and not both. Table II gives the retention times for the various naphthoquinones. As can be seen on system A clean separations of MK-4, MK-3 and their epoxy derivatives can be achieved and in particular MK-3 is separated well from epoxy MK-4. On system B phylloquinone and epoxyphylloquinone are readily separable. In an earlier study a similar separation of phylloquinone and epoxyphylloquinone was achieved on a 40-cm column of Permaphase ODS using methanol–water

TABLE II

RETENTION TIMES FOR VITAMIN K AND DERIVATIVES ON HPLC

Retention times are given in minutes.

Compound	*HPLC system*	
	*A**	*B***
Menaquinone-4	27.5	
2,3-Epoxymenaquinone-4	20.0	
Menaquinone-3	17.0	
2,3-Epoxymenaquinone-3	12.0	
Phylloquinone		9.6
2,3-Epoxyphylloquinone		7.2
Menadione	5.0	

* 3 × 30 cm μBondapak C_{18} columns, acetonitrile–water (85:15) as solvent.

** 2 × 20 cm μBondapak C_{18} columns, methanol as solvent.

(90:10) as solvent[14]. Separations involving menaquinones were not attempted in that study.

Both systems *i.e.* TLC and HPLC have their advantages and disadvantages. Sensitivity is much greater in the case of HPLC where as little as 5–10 ng of vitamin K could be detected whereas a comparable figure on TLC was 250–500 ng. Speed was found to be similar, from 30 to 45 min in each case but of course in the case of TLC a quantitative estimation has to be carried out after the separation whereas it is concurrent in HPLC. However, to reach a level of separation in TLC which is comparable to that obtained with HPLC at least two successive TLC systems must be used. To separate for example MK-3, MK-4 and epoxy MK-4 by TLC, the mixture must be applied to reversed-phase TLC initially which will separate MK-4 from MK-3 and epoxy MK-4 which migrate together. The two bands are eluted from the TLC plate and re-chromatography on silica gel G plates using light petroleum as solvent separates the vitamin K fractions from contaminating paraffin (vitamin K fractions stay at the origin and the paraffin migrates up the plate). After elution of the mixed MK-3 and epoxy MK-4 they are applied to an argentation plate when the final separation is made. Rather more material can be applied to a thin-layer chromatogram than to an HPLC column. In our experience the maximum amount of a mixture which can successfully be applied to an HPLC column is 500 μg whereas perhaps 1 mg can be applied as a 15-cm line on a thin-layer chromatogram.

Reversed-phase or argentation TLC on the one hand and HPLC on the other both need some prior purification before they can be used successfully. It is totally impossible to apply crude lipid fractions to these systems, not only is it difficult applying sufficient lipid to allow detection of the vitamin K but also the presence of other lipids can cause distortion in the chromatogram. If a crude lipid is chromatographed on an adsorption thin-layer system, *e.g.* 6% diethyl ether in light petroleum (see Table I) a broad band from R_F 0.27 to 0.35 will contain the whole vitamin K series of compounds discussed here. Elution of this band then allows that fraction to be used for HPLC or further TLC systems.

To confirm the viability of the HPLC method system C (Fig. 1) was used to study the incorporation of [2-^{14}C]mevalonate into vitamin K in *Carcinus maenas*. When unlabelled menadione was administered together with the [2-^{14}C]mevalonate radioactivity was detected in MK-4 and epoxy MK-4 following HPLC and when menadione was replaced by naphthoquinone radioactivity was found mainly in demethyl MK-4 with smaller amounts in MK-4 and epoxy MK-4.

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