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Note

Resolution of phylloquinone (vitamin K_1), phylloquinone 2,3-epoxide, 2-chloro-phylloquinone and their geometric isomers by high-performance liquid chromatography

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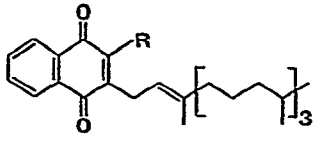
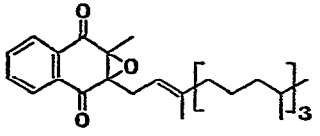
Developments in the chromatography of the K vitamins have played an important role in current understanding of the function of these isoprenoid quinones in the carboxylation of peptide-bound glutamyl residues to γ-carboxyl glutamyl residues. For example, the discovery of the metabolic interconversion of phylloquinone (vitamin K_1, structure I) and phylloquinone 2,3-epoxide (vitamin K_1 epoxide, structure III) by Matschiner and co-workers[1] was made possible by earlier developments of reversed-phase chromatographic techniques for the separation of menaquinones (K_2 vitamins)[2–4]. To date most studies requiring the resolution of K_1 and K_1 epoxide have used reversed-phase thin-layer chromatography (TLC) although their resolution by reversed-phase high-performance liquid chromatography (HPLC) has also been reported[5–7]. We have briefly reported a more efficient separation of K_1 and K_1 epoxide using non-aqueous reversed-phase HPLC[8] and in this paper we describe in detail the chromatography of these compounds by both adsorption and reversed-phase modes of HPLC.



I. phylloquinone (R=CH_3)

II. 2-chloro phylloquinone (R=Cl)



III. phylloquinone 2,3-epoxide

Another aspect of the chromatography of vitamin K_1 and related compounds for which we considered that HPLC would be superior to other methods is for the resolution of *cis*- and *trans*-isomers at the 2′,3′ position of the phytyl side-chain. Previously these isomers have been purified by conventional column chromatography or by TLC[9–12]. These methods are time-consuming because the resolution

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of the isomers is incomplete and repeated chromatography is necessary to achieve a high isomeric purity.

Because of its close structural similarity to vitamin K_1 and its experimental use as a vitamin K antagonist[13,14] we have also studied the chromatographic behaviour of the compound 2-chloro-3-phytyl-1,4-naphthoquinone (2-chloro-phylloquinone, structure II).

EXPERIMENTAL

Chemicals

Synthetic phylloquinone (K_1) was a gift from Hoffmann-La Roche, Basle, Switzerland. Phylloquinone 2,3-epoxide (K_1 epoxide) was synthesised from K_1 by the method of Tishler *et al.*[15]. 2-Chloro-phylloquinone (chloro-K_1) was obtained as a gift from Dr. R. G. Bell, University of Rhode Island, U.S.A. and from Dr. M. G. Townsend, Pest Infestation Control Laboratory, Tolworth, Great Britain. HPLC grade solvents were obtained from Rathburn Chemicals, Walkerburn, Great Britain. The dichloromethane was free of stabiliser. The 50% water-saturated dichloromethane was prepared by mixing equal volumes of dry and fully water-saturated solvent.

High-performance liquid chromatography

Pumps were either reciprocating (Model 750/03) or constant pressure (Model 750/01) both obtained from Applied Chromatography Systems, Luton, Great Britain. These were coupled to a variable wavelength UV detector (Model LC from Pye Unicam, Cambridge, Great Britain) operated at 250 or 270 nm.

The packing materials and dimensions of columns used for HPLC were as follows: Spherisorb-5 (250 × 5 mm I.D.), and Partisil-5 (250 × 4.9 mm I.D.), Zorbax-ODS (250 × 4.6 mm I.D.), Spherisorb-5-ODS (250 × 5 mm I.D.), and Hypersil-ODS (100 × 5 mm I.D.). Spherisorb-5 and Spherisorb-5-ODS packings were supplied by Phase Separations, Queensferry, Great Britain and packed into columns by HPLC Technology, Wilmslow, Great Britain. Partisil-5 packing was supplied by Whatman, Maidstone, Great Britain, and the column packed by Hichrom, Woodley, Great Britain. Hypersil-ODS packing was obtained from Shandon-Southern Instruments, Runcorn, Great Britain, and the column packed in our laboratory according to the directions of the manufacturers. The Zorbax-ODS column was obtained pre-packed from DuPont, Hitchin, Great Britain.

Samples were dissolved in the mobile phase and injected with a syringe and on-column septum injector (HPLC Technology) except for the Partisil-5 column which was fitted with a syringe loading injection valve (Model 7125) from Rheodyne, Berkeley, CA, U.S.A.

RESULTS

Adsorption HPLC

Table I shows the capacity ratios for *cis*- and *trans*-isomers of K_1, K_1 epoxide and chloro-K_1 for systems of adsorption HPLC on two commercial packings which had silica particles of 5-μm diameter and either an irregular shape (Partisil-5) or a

TABLE I

CAPACITY RATIOS OF *CIS*- AND *TRANS*-ISOMERS OF VITAMIN K COMPOUNDS BY ADSORPTION HPLC

Compound	*Mobile phase, column and flow-rate*				
	0.5% Acetonitrile in n-hexane		*25% Dichloromethane (50% water-saturated) in n-hexane*		*25% Dichloromethane (dry) in n-hexane, Spherisorb-5 (2 ml/min)*
	Partisil-5 (1 ml/min)	*Spherisorb-5 (1 ml/min)*	*Partisil-5 (2 ml/min)*	*Spherisorb-5 (1 ml/min)*	
cis-Cl-K_1	1.2*	1.1*	1.8	1.0	2.8
trans-Cl-K_1	1.2*	1.2	2.2	1.3*	3.5
cis-K_1	1.1	0.9	2.7	1.3*	4.4
trans-K_1	1.2*	1.1*	3.3*	1.7	5.7
cis-K_1 epoxide	1.6**	1.4**	3.3*	1.4**	4.7
trans-K_1 epoxide	1.6**	1.4**	3.3*	1.4**	5.0

*,** Denotes compounds not resolved from each other.

spherical shape (Spherisorb-5). Baseline resolution of *cis*- and *trans*-isomers of K_1 and chloro-K_1 was readily achieved on both packings by mobile phases of dichloromethane in hexane. Mobile phases containing a very low concentration of a polar moderator such as acetonitrile were less effective in resolving these isomers. Originally when employing dichloromethane as a moderator we controlled the activity of the silica adsorbent by preparing dichloromethane that was 50% water-saturated. Later we observed that the resolution between *cis*- and *trans*-isomers was improved by employing dry dichloromethane, although the 50% water-saturated solvent gave higher column efficiencies. As illustrated by the chromatogram in Fig. 1, a column of Spherisorb-5 with a mobile phase of dry dichloromethane in hexane resolved all six isomeric forms contained in a mixture of K_1, chloro-K_1 and K_1 epoxide. This system was the only one found that could resolve *cis*- and *trans*-isomers of K_1 epoxide ($R_s = 1.1$) and in addition gave little or no overlap of isomers from different compounds (Table I).

An interesting finding was that the order of elution of the pair of compounds K_1 and K_1 epoxide depended on the moderator employed. Thus with a mobile phase of 0.5% acetonitrile in hexane, K_1 eluted before K_1 epoxide on both Partisil-5 and Spherisorb-5 though only K_1 was further resolved into its *cis*- and *trans*-isomers (Table I). In contrast K_1 epoxide tended to be eluted before K_1 with mobile-phase mixtures of dichloromethane and hexane. On Partisil-5 this effect was slight so that *trans*-K_1 and K_1 epoxide (*cis*- and *trans*-isomers unresolved) eluted in the same peak whilst, on Spherisorb-5, K_1 epoxide eluted before *trans*-K_1 giving peaks that were well resolved (Table I).

Reversed-phase HPLC

The chromatography of K_1 and related compounds was examined on three different commercial packings in which an ODS phase was bonded to 5-μm porous silica particles. Although each column required a totally organic mobile phase, the solvent composition required to obtain similar capacity ratios varied considerably

TABLE II

CAPACITY RATIOS (k'), SELECTIVITY (α) AND RESOLUTION (R_s) OF VITAMIN K COMPOUNDS BY REVERSED-PHASE HPLC

Compound	*Mobile phase, column and flow-rate*				
	Acetonitrile, 100%, Hypersil-ODS (1.0 ml/min)	*Dichloromethane in acetonitrile*		*Dichloromethane in methanol*	
		5%, Spherisorb-5-ODS (1.4 ml/min)	*30%, Zorbax-ODS (0.8 ml/min)*	*5%, Spherisorb-5-ODS (1.2 ml/min)*	*20%, Zorbax-ODS (1.1 ml/min)*
k'					
K_1 epoxide	2.5	1.9	1.7	1.6	1.7
Cl-K_1	3.9	2.8	2.5	2.5*	2.6*
K_1	4.4	3.1	2.8	2.5*	2.6*
α					
K_1/K_1 epoxide	1.8	1.6	1.6	1.6	1.5
K_1/chloro-K1	1.1	1.1	1.1	1.0	1.0
R_s					
K_1/chloro-K_1	1.1	0.8	1.6	—	—

* Denotes compounds not resolved from each other.

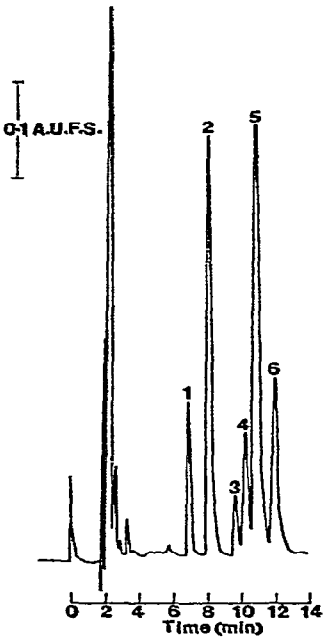

Fig. 1. Separation of *cis*- and *trans*-isomers of vitamin K compounds by adsorption HPLC on Spherisorb-5 with a mobile phase of 25% dichloromethane (dry) in hexane and a flow-rate of 2 ml/min. Detection was at 250 nm. Peaks: 1 = *cis*-chloro-K_1; 2 = *trans*-chloro-K_1; 3 = *cis*-K_1; 4 = *cis*-K_1 epoxide; 5 = *trans*-K_1 epoxide; 6 = *trans*-K_1.

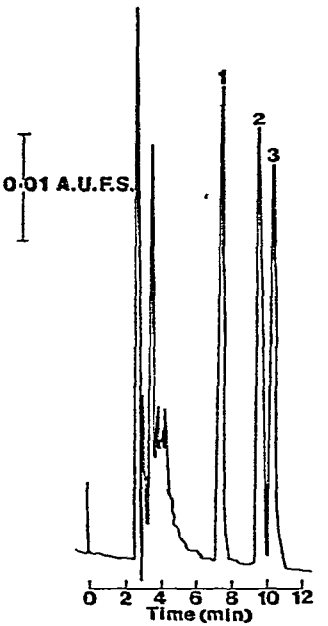

Fig. 2. Separation of vitamin K compounds by reversed-phase HPLC on Zorbax-ODS with a mobile phase of 30% dichloromethane in acetonitrile at a flow-rate of 0.8 ml/min. Detection was at 270 nm. Peaks: 1 = K_1 epoxide; 2 = chloro-K_1; 3 = K_1.

(Table II). This difference in retention was attributed to the different degrees of carbon-loading of the supports. Thus, whilst chromatography of the compounds on Hypersil-ODS (column length 10 cm) was conveniently achieved with pure acetonitrile or methanol, columns packed with Spherisorb-5-ODS or Zorbax-ODS (column length 25 cm) required the further addition of a less polar solvent such as dichloromethane. Although all three ODS-bonded phases were equally effective in resolving K_1 and K_1 epoxide with mobile phases based on either acetonitrile or methanol, the resolution of K_1 from chloro-K_1 was only possible with mobile phase containing acetonitrile (Table II). A representative chromatogram illustrating the resolution of all three compounds on Zorbax-ODS is shown in Fig. 2. This support, which was also the most retentive, gave the best resolution of K_1 and chloro-K_1 ($R_s = 1.6$).

DISCUSSION

With increased knowledge of the biological significance of vitamin K_1 epoxide, improved chromatographic methods for the separation of this metabolite from vitamin K_1 should be of value, especially for the assay of the two enzymes responsible for their interconversion[5]. The microparticulate ODS phases employed in our studies are ideally suited to the efficient resolution of K_1 and K_1 epoxide with non-aqueous mobile phases. The principles and advantages of non-aqueous over semi-

aqueous mobile phases for the reversed-phase separations of non-polar compounds on highly retentive ODS phases have been enumerated by Parris[16]. These advantages, which extend to the analysis of K vitamins, include the enhanced solubility of the sample in the mobile phase, lower operating pressures, high column efficiencies and in our experience an extended column life.

Although reversed-phase HPLC gave the best resolution of K_1 and K_1 epoxide they could also be resolved by silica supports. The elution of K_1 before K_1 epoxide with acetonitrile as a moderator is that expected for an adsorption mechanism since K_1 epoxide is slightly more polar than K_1. In contrast the elution of K_1 after K_1 epoxide with dichloromethane as a moderator on Spherisorb-5 silica was the same order as found on reversed-phase columns, although the silica column retained the characteristic ability of adsorption systems to resolve *cis*- and *trans*-isomers.

Since natural K_1 is composed only of the *trans*-isomer, whilst synthetic K_1 contains a mixture of *cis*- and *trans*-isomers[9,17], the separation of these isomers is sometimes required on a preparative scale, especially for studies of their biological activity[10–12] or metabolism[18]. Previously the purification of *cis*- and *trans*-isomers of K_1 by conventional liquid chromatography or TLC has resulted in preparations with different purities which has led to conflicting conclusions about their relative biological activities[10–12]. The systems of adsorption HPLC developed here offer a more efficient method of purifying these isomers. Besides the greater resolution of HPLC, as little as 500 pg of a contaminating isomer could be detected photometrically, an amount which is *ca.* 1000-fold less than could be detected when the isomers were purified by TLC[12].

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NOTE ADDED IN PROOF

In a recent paper[19] describing an assay of vitamin K_1 in serum, the authors also discuss the resolution of K_1 and K_1 epoxide on packings based on 5-μm Rsil silica (RSL, St. Martens-Latem, Belgium).

In adsorption HPLC using acetonitrile as a moderator in hexane, Rsil silica gave a similar order of elution and resolution of *cis*-K_1, *trans*-K_1 and K_1 epoxide as found in our studies with Partisil-5 and Spherisorb-5 silicas. Lefevere *et al.*[19], however, found a lower selectivity of dichloromethane over acetonitrile on Rsil with a complete loss of resolution of the geometric isomers of K_1 and also of K_1 from K_1 epoxide. This is in contrast to our findings with Spherisorb-5 where dichloromethane increased the selectivity for the resolution of the geometric isomers (Fig. 1). Although the resolution of K_1 and K_1 epoxide was lost with mixtures of dichloromethane in hexane on both Partisil and Rsil silicas these compounds could still be resolved on Spherisorb-5. The eluting order of K_1 and K_1 epoxide however from Spherisorb-5 was the reverse of that expected on the basis of their polarity (Table I). These differences between packings may be related to their particle shape since the silica particles of Partisil-5 and Rsil are irregular whilst those of Spherisorb-5 are spherical.

Our findings for the reversed-phase separation of K_1 and K_1 epoxide were in close agreement with those of Lefevere and co-workers who employed Rsil C_8 and C_{18} phases.

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