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Note

Quantitative analysis of pharmacological levels of vitamin K_1 and vitamin K_1 2,3-epoxide in rabbit plasma by high-performance liquid chromatography

ADRIAN C. WILSON and B. KEVIN PARK*

Department of Pharmacology and Therapeutics, University of Liverpool, Ashton Street, Liverpool L69 3BX (U.K.)

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Vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone) is a cofactor for the post-ribosomal γ-carboxylation of glutamyl residues in clotting factors II, VII, IX and X. Carboxylation of the glutamyl residues imparts calcium binding properties to these enzymes. During clotting factor precursor γ-carboxylation, vitamin K_1 is metabolised to vitamin K_1 2,3-epoxide [1—4]. The epoxide metabolite is reconverted back to vitamin K_1 by vitamin K_1 epoxide reductase. It has been proposed a cyclic interconversion of the vitamin and the epoxide, termed the vitamin K_1 K_1 epoxide cycle, is thought to have a role in preserving the body pool of the vitamin. Recent advances in vitamin K_1 biotransformations have been well reviewed [5, 6]. The interconversion of vitamin K_1 and its epoxide occurs in the liver and can be inhibited by coumarin anticoagulants such as warfarin [7, 8], acenocoumarin [9] and novel derivatives such as difenacoum [10].

Investigation of vitamin K_1, its metabolism and interaction with anticoagulants, has been carried out primarily in the rat [4, 11, 12] although recently the rabbit has been shown to be a good animal model with which to study the temporal changes and modifications of vitamin K_1 metabolism in vivo [13—15]. However, in these studies it was necessary to use radiolabelled vitamin K_1 and thin-layer chromatography (TLC) as a separative procedure to identify K_1 metabolites in biological fluids. This rather cumbersome method was used because of the lack of an alternative, reliable assay for vitamin K_1.

High-performance liquid chromatography (HPLC) has recently been developed to separate and quantify the various forms of vitamin K present in human biological fluids and green vegetables [16—19]. Extremely sensitive two-column HPLC systems have been developed by Lefevere and co-workers [19, 20] to measure physiological levels of vitamin K_1 in human serum with a

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sensitivity of 0.9 ng/ml. These methods involve fractionation of serum extracts using a silica adsorption column followed by reversed-phase separation of the appropriate reconstituted eluate on a C18 column. However, to continue our research we required a rapid, reproducible and reliable assay for vitamin K_1 and its metabolites in rabbit plasma after administration of a pharmacological dose of the vitamin. For this purpose it was only necessary to use a single-column HPLC separation because of the relatively high levels of vitamin K_1 in the plasma extracts and in order to reduce the assay time in view of the large number of samples measured. We report here both normal- and reversed-phase HPLC methods developed for this purpose.

EXPERIMENTAL

Apparatus

HPLC was carried out using the following components: an Altex 110A isocratic solvent delivery pump, an Altex 160 fixed-wavelength UV detector connected to a Gilson N1 potentiometric recorder. Glassware was first rinsed with 5% dimethyldichlorosilane in toluene and thereafter methanol-washed.

Reagents

Chemicals and reagents used were of analytical grade. All solvents used were HPLC grade (Rathburn Chemicals, Walkerburn, U.K.). Vitamin K_1 2,3-epoxide was synthesised by the method of Tishler et al. [21], the structure confirmed by UV absorbance between 200 and 400 nm and its purity verified by normal-phase HPLC. No residual vitamin K_1 was detected. Vitamin MK4 (2-methyl-3-farnesylfarnesyl-1,4-naphthoquinone) was a gift from Hoffman-La Roche (Basle, Switzerland). 2-Chloro-3-phytyl-1,4-naphthoquinone (Cl-K) was a gift from Sorex Laboratories (Widnes, U.K.).

Standard solutions

Solutions of 5, 50 and 500 μg/ml of vitamin K_1, its 2,3-epoxide, MK4 and Cl-K were prepared in hexane and stored protected from fluorescent light.

Blood sampling

Male New Zealand White rabbits (2.5—3.0 kg) were injected intravenously into the marginal ear vein with 1 mg/kg of phylloquinone (Konakion®) and blood was collected from the opposite ear at times 0.25, 0.5, 0.75, 1, 2, 3, 4, 5 and 6 h. Blood samples were centrifuged (2000 *g*) and plasma stored at −20°C until required for assay purposes.

Plasma extraction

An aliquot of the internal standard solution (either vitamin MK4 or Cl-K) was put into a 10-ml glass tube and blown dry under nitrogen. Plasma (1 ml) was then added, the tube vortexed for 30 sec and left at room temperature to equilibrate for 15 min. Following equilibration of the plasma an equal volume of methanol was added and the tube shaken mechanically for 2 min. Hexane (5 ml) was added and the tube shaken again for a further 5 min. To ensure complete separation of the methanol—water phase from hexane, the tube was centrifuged for 1 min at 500 *g*. The hexane layer was removed, blown dry

under nitrogen and redissolved in 100 μl of the appropriate eluent. A 20-μl volume of this solution was injected into the chromatograph.

Chromatographic conditions

Reversed phase. The mobile phase used was 30% dichloromethane in acetonitrile which was degassed by sonication prior to use; the flow-rate was 1 ml/min with a pressure of 35- 100 bar, UV detection was effected at 254 nm at a sensitivity of up to 0.002 a.u.f.s.; quantification was by peak height comparison. Calibration graphs were calculated from the linear regression curves obtained from the ratio of the peak heights of 2-chlorovitamin K_1 to that of either vitamin K_1 or vitamin K_1 2,3-epoxide at concentrations over the pharmacological range observed in rabbit plasma. A reversed-phase column was used for the separation (Ultrasphere ODS C18 with 5 μm particle diameter, 25 cm × 4.5 mm I.D., Altex, Berkeley, CA, U.S.A.) protected by a guard column (2.5 cm × 4.5 mm I.D.) packed with Ultrasphere ODS C18, particle size 5 μm. Column efficiency was ⩾ 55,000 plates/m for the test compounds including the internal standard.

Normal phase. The mobile phase used in this system was 0.2% acetonitrile in hexane pumped at 2 ml/min (35—100 bar). UV detection was effected at 254 nm with a sensitivity of up to 0.002 a.u.f.s. Calibration curves were calculated as for the reversed-phase system but using vitamin MK4 as internal standard. The normal-phase system used a Partisil-10 column (25 cm × 4.5 mm I.D., 10 μm particle diameter, Whatman, Maidstone, U.K.) protected by a guard column (2.5 cm × 4.5 mm I.D.) packed with Partisil-10 silica gel. This guard column was repacked at 3-monthly intervals when column efficiency was seen to decline. Normal-phase column efficiency was typically greater than 20,000 plates/m for all test compounds including the internal standard. Both normal- and reversed-phase separations were run at ambient temperature.

RESULTS

Reversed phase

Fig. 1a shows a typical chromatographic separation of vitamin K_1, its 2,3-epoxide and Cl-K (internal standard).

Retention times were 8.2 min for vitamin K_1 2,3-epoxide, 10.5 min for Cl-K and 11.1 min for vitamin K_1 giving a run time of 15 min. Fig. 1b shows a rabbit extract taken 2 h after 1 mg/kg phylloquinone was administered. There was slight interference of the vitamin K_1 2,3-epoxide peak with background UV absorbing material. The calculated recoveries from plasma were 94.7 ± 4.3% for vitamin K_1, 91.7 ± 3.6% for vitamin K_1 2,3-epoxide and 94.1 ± 5.1% for Cl-K at a plasma concentration of 0.5 μg/ml. Plasma analysis was in the range of 0.1—10 μg/ml with a sensitivty of 100 ng/ml.

Linear regression lines obtained from the standard graphs were $y = 1.8739x + 0.0046$, $r = 0.993$ for vitamin K_1 and $y = 0.6543x + 0.0738$, $r = 0.997$ for vitamin K_1 2,3-epoxide. Intra-assay variation was calculated by the repeated chromatographing of a single-spiked plasma sample and gave a coefficient of variation of 3.9%. The coefficient of variation for the calculated slopes of the standard graphs was 7.7% ($n = 6$).

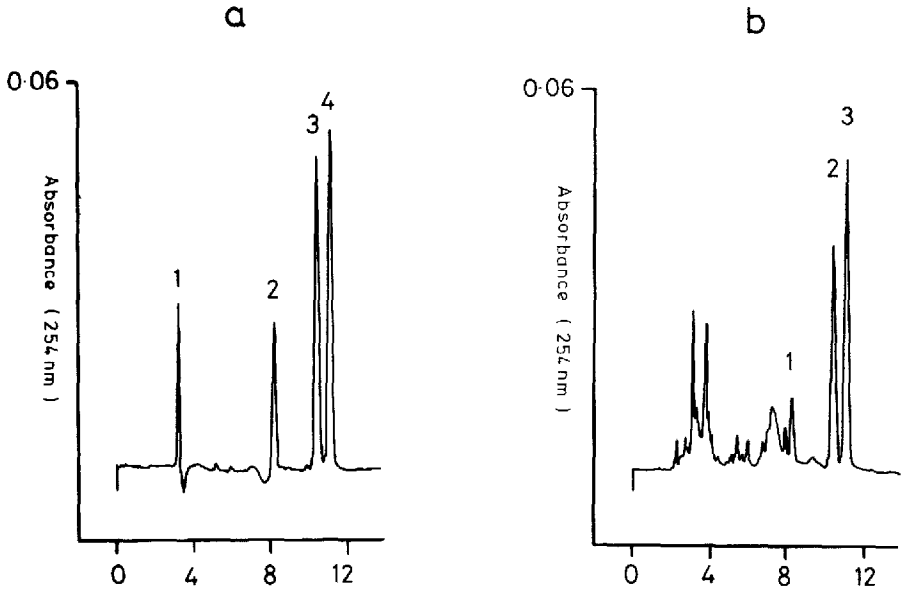

Fig. 1. Reversed-phase high-performance liquid chromatograms. (a) Separation of 300 ng vitamin K_1 (4), vitamin K_1 2,3-epoxide (2) and Cl-K (3); (1) is the solvent front. (b) Extract of 1 ml rabbit plasma taken 2 h following intravenous administration of phylloquinone (1 mg/kg). Peaks: 1 = vitamin K_1 2,3-epoxide; 2 = internal standard, Cl-K (1 μg/ml); 3 = vitamin K_1.

Normal phase

Fig. 2A shows a typical chromatographic separation of vitamin K_1, its 2,3-epoxide and MK4. Vitamin K_1 exists in the *cis* and *trans* isomeric forms at the 2′,3′ position of the phytyl side-chain. It can be seen that normal-phase HPLC clearly separates these geometric isomers of vitamin K_1 with retention times of 6.9 min for *cis*-vitamin K_1 and 7.8 min for *trans*-vitamin K_1. The retention time for vitamin K_1 2,3-epoxide was 8.7 min and for vitamin MK4, 11 min. Fig. 2B shows a chromatogram of rabbit plasma taken 4 h following intravenous administration of 1 mg/kg phylloquinone. There was no interference from UV absorbing material in rabbit plasma at sensitivities below 0.005 a.u.f.s. Plasma recoveries were greater than 90% for all standards at a concentration of 0.2 μg/ml and plasma analysis was in the range 0.02–10 μg/ml giving a sensitivity of 20 ng/ml. Linear regression lines were obtained from the standard graphs and were $y = 0.3597x + 0.0101$, $r = 0.994$ for *cis*-vitamin K_1, $y = 0.8939x + 0.0185$, $r = 0.993$ for *trans*-vitamin K_1 and $y = 0.4180x + 0.0284$, $r = 0.995$ for vitamin K_1 2,3-epoxide. Intra-assay variation, calculated from repeated sampling of a single-spiked plasma sample, gave a coefficient of variation of 3.6%. The coefficient of variation of the slopes of the standard graphs, calculated over a two-month period, was 7.0%.

Fig. 3 shows levels of vitamin K_1 in rabbits given 1 mg/kg phylloquinone intravenously. Plasma concentration—time curves were obtained for comparison using both reversed-phase and normal-phase HPLC. The plasma profiles are similar to those obtained using radiolabelled phylloquinone [15].

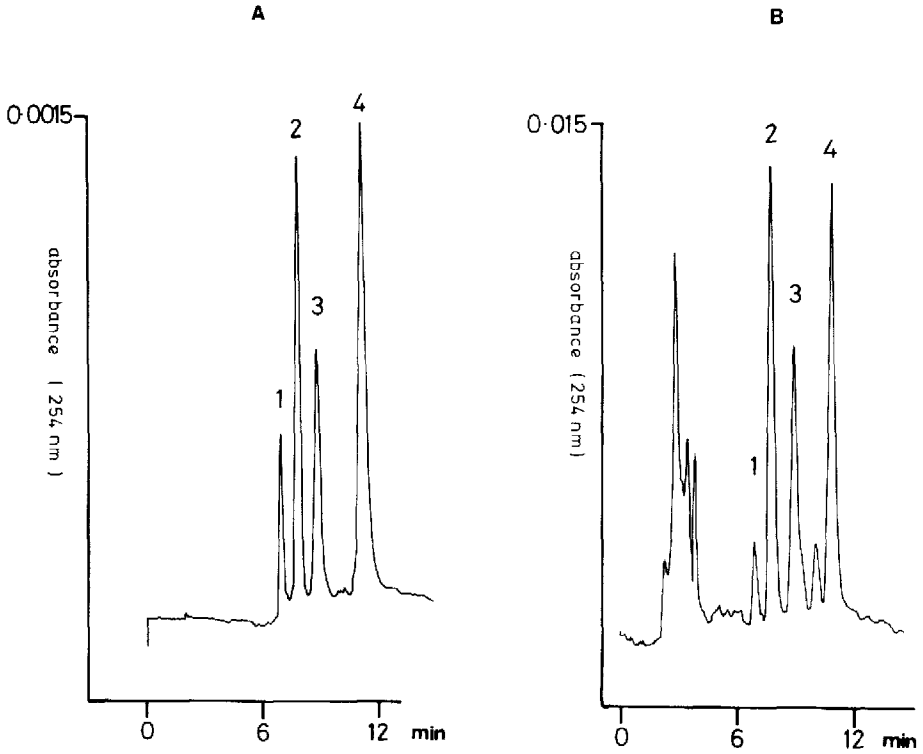

Fig. 2. Normal-phase high-performance liquid chromatograms. (A) 23 ng Vitamin K_1 (separation of the *cis* and *trans* isomers), vitamin K_1 2,3-epoxide and vitamin MK4. (B) Extract of 1 ml rabbit plasma taken 4 h after intravenous administration of phylloquinone (1 mg/kg). Peaks: 1 = *cis*-vitamin K_1; 2 = *trans*-vitamin K_1; 3 = vitamin K_1 2,3-epoxide; 4 = vitamin MK4 (internal standard added to a concentration of 0.5 μg/ml).

Commercial vitamin K_1 contains a mixture of the *cis* and *trans* geometric isomers that range from 12% *cis*-vitamin K_1 in Konakion (Hoffmann-La Roche, Nutley, NJ, U.S.A.) to 28% in phylloquinone (Sigma, St. Louis, MO, U.S.A.). Photoisomerisation of the *trans* to *cis* form of vitamin K_1 occurs especially in fluorescent light. A sample of chromatographically pure *trans*-vitamin K_1 underwent 20% isomerisation when exposed to fluorescent light for 40 h. As a precautionary measure plasma samples and solvent extracts were protected from light at all times by wrapping the tubes in aluminium foil.

DISCUSSION

The two HPLC assays reported here, the normal- and reversed-phase systems, both show good separation of vitamin K_1, its 2,3-epoxide metabolite and the internal standards used. The normal-phase HPLC system, however, is superior to the reversed-phase one because of its ability to separate the *cis* and *trans* isomers of vitamin K_1.

Cis-vitamin K_1 has far less biological activity than *trans*-vitamin K_1 in stimulating prothrombin synthesis and vitamin K_1 2,3-epoxide formation [22].

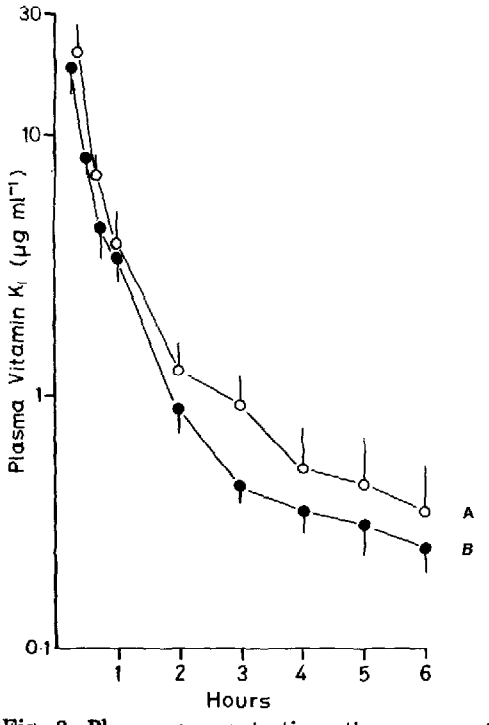

Fig. 3. Plasma concentration—time curves for vitamin K_1 in rabbit plasma following intravenous administration of phylloquinone (1 mg/kg). All vitamin K_1 levels were obtained using (A) reversed-phase and (B) normal-phase HPLC. Plasma levels in graph B refer to the sum of the *cis* and *trans* isomer of vitamin K_1. Values are given as mean ± S.D. (n = 4).

Also, following injection of a mixture of the *cis* and *trans* isomers, the disappearance of *cis*-vitamin K_1 from all subcellular fractions of rat liver is slower than that of the *trans* isomer [23]. Hence, for our research in the pharmacokinetics of vitamin K_1 and perturbations caused by anticoagulants, we require to measure and monitor the plasma decline of the active *trans* form of the vitamin following intravenous administration of the commercial preparations of vitamin K_1.

A previous normal-phase HPLC assay developed to separate and quantify endogenous physiological amounts of the geometric isomers of vitamin K_1 required a two-column system [19]. This assay, although very sensitive in its detection limit (500 pg/ml plasma), requires a complicated analytical procedure. The normal-phase system reported here, for the analysis of vitamin K_1 in rabbit plasma following pharmacological doses of the vitamin, is a simple single extraction, single-column chromatographic procedure which is rapid and affords high sensitivity. Interference with background UV absorbing material co-extracted from rabbit plasma is minimal and the normal-phase HPLC method quantitates 3 ng *trans*-vitamin K_1 injected on column. This system can be used for the rapid and routine analysis of plasma from the rabbit; an ideal animal model for the study of temporal changes to vitamin K_1 metabolism in vivo.

CONCLUSION

The determination of vitamin K_1 and vitamin K_1 2,3-epoxide, after single hexane extraction, was effected through isocratic HPLC. Both reversed-phase and normal-phase systems were used, the former using a C18 column as stationary phase with dichloromethane and acetonitrile as eluent and the latter a silica column with acetonitrile—hexane as the mobile phase. UV detection was at 254 nm; chlorovitamin K was used as internal standard for reversed-phase HPLC while the normal-phase system used vitamin K_2(MK4).

Normal-phase HPLC was found to be superior to the reversed-phase system with respect to the lack of interference from UV absorbing material co-extracted with vitamin K_1 and its 2,3-epoxide from rabbit plasma. The methods were used in the range 0.02—10 μg/ml, the sensitivity was 20 ng/ml for the normal phase system and the precision of the assay was in the range 3—7%. Plasma levels were measured using both HPLC systems as a comparison in rabbits after a pharmacological dose of vitamin K_1.

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