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Note

Quantitative analysis of pharmacological concentrations of vitamin K_1 and vitamin K_1 2,3-epoxide in rat liver by high-performance liquid chromatography

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Vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone) is a co-factor for the post-ribosomal γ-carboxylation of glutamyl residues in clotting factors II, VII, IX and X [1]. During γ-carboxylation vitamin K_1 is converted to vitamin K_1 2,3-epoxide which has no biological activity [2]. The enzyme vitamin K_1 epoxide reductase is responsible for the reduction of the epoxide back to vitamin K_1 This cycle of events is referred to as the vitamin K_1 epoxide cycle and takes place in the liver Coumarin anticoagulants such as warfarin [3, 4], acenocoumarin [5] and novel derivatives such as brodifacoum [6] are thought to inhibit clotting factor synthesis by inhibition of the epoxide reductase enzyme.

Although clotting factor synthesis occurs in the liver, much of the information previously obtained concerning the disposition and mode of action of vitamin K_1 has been based on plasma concentration data [7, 8] As a consequence several sensitive and selective methods for the measurement of vitamin K_1 in plasma have been developed [9, 10]

Analysis of hepatic concentrations of vitamin K_1 and its metabolites has been restricted to radiometric methods [11] The measurement of endogenous vitamin K_1 alone has been achieved using multi-stage assays with either ultraviolet [12] or more recently electrochemical detection [13] However, the simultaneous determination of vitamin K_1 2,3-epoxide was not described and therefore these methods were not suitable for our pharmacological investigations

We report here a sequential chromatographic method developed to determine hepatic concentrations following administration of pharmacological doses of the vitamin or its epoxide. The assay enabled us to accurately and routinely

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determine the hepatic content of vitamin K_1 and vitamin K_1 2,3-epoxide in control and anticoagulant pretreated animals.

EXPERIMENTAL

Apparatus

Normal-phase high-performance liquid chromatography (HPLC) was carried out using an Altex 110A isocratic solvent delivery pump, an Altex 160 fixed-wavelength UV detector connected to an N1 potentiometric recorder Reversed-phase HPLC was carried out using a Spectra-Physics SP 8700 solvent delivery system, a Kratos (Spectraflow 773) fixed-wavelength UV detector connected to a Philips PM-8251 single-pen recorder. Glassware was rinsed with 5% dimethyldichlorosilane in toluene and thereafter washed with methanol

Reagents

Chemicals and reagents used were of analytical grade. Vitamin K_1 2,3-epoxide was synthesized by the method of Tischler et al [14], and its purity verified by normal-phase HPLC No residual vitamin K_1 was detected. Warfarin was obtained from Ward Blenkinsop (Widnes, U K.). Vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone) was obtained from Sigma (St. Louis, MO, U S.A.). Brodifacoum and 2-chloro-3-phytyl-1,4-naphthoquinone (Cl-K) were gifts from Sorex Labs (Widnes, U.K.). The acetone used was from Koch-Light Labs (Colnbrook, U K). All other solvents used were HPLC grade from Fisons (Loughborough, U K)

Standard solutions

Solutions of 5, 50 and 500 μg ml^{-1} vitamin K_1, vitamin K_1 2,3-epoxide and Cl-K in hexane were prepared for normal-phase HPLC The same concentrations were prepared in acetonitrile for reversed-phase HPLC All standard solutions were stored protected from fluorescent light

Liver preparation

Male Wistar rats (210—260 g) were anaesthetised using urethane (14% solution 1 ml 100 mg^{-1}) and each rat received 1 mg kg^{-1} vitamin K_1 (Konakion®) or vitamin K_1 2,3-epoxide dissolved in Tween 80 and diluted with 0.9% saline to make solutions containing 5% Tween via a jugular vein cannula Those rats receiving warfarin (63 mg kg^{-1}) in 0.9% (w/v) sodium chloride solution (1.0 ml kg^{-1}) or brodifacoum (10 mg kg^{-1}) in polyethylene glycol 200 (1 ml kg^{-1}) were pretreated intraperitoneally (i.p) with anticoagulant 6 h before administration of vitamin K_1 or vitamin K_1 2,3-epoxide A cannula was inserted into the hepatic portal vein 3 h after receiving the vitamin or its epoxide and the liver perfused with phosphate buffer (2 ml) The liver was subsequently removed, homogenised in 2 vols. phosphate buffer and frozen (−30°C) until required for analysis

Liver extraction

To an aliquot of liver homogenate (100 μl) internal standard, Cl-K (400—1000 ng), was added, the suspension vortexed for 0.5 min and left at

room temperature to equilibrate for 15 min Acetone (0 5 ml) was then added and the mixture mechanically shaken for 20 min Hexane (2 ml) was added, the contents of the tube shaken for a further 10 min and then centrifuged (5000 *g*, 5 min) to ensure complete separation of the organic layer from the aqueous layer The organic layer was removed, evaporated under vacuum at 50°C and redissolved in 60 μl of normal-phase eluent Following the injection of 50 μl of this solution onto the normal-phase HPLC system, the effluent was collected between 6 and 10 min to ensure complete recovery of vitamin K_1, vitamin K_1 2,3-epoxide and the internal standard (Fig. 1). Under vacuum at 50°C the effluent was evaporated and then redissolved in 100 μl of reversed-phase eluent, 50 μl of this solution were injected onto the reversed-phase system for analysis

For *cis*- and *trans*-vitamin K_1 determination, the effluent corresponding to the vitamin K_1 peak during reversed-phase HPLC was collected, evaporated under vacuum at 50°C, redissolved in 100 μl of normal-phase eluent and 50 μl injected onto column

Chromatographic conditions

Normal phase The mobile phase was 0 23% acetonitrile in hexane which was degassed by sonication prior to use, the flow-rate was 2 ml min^{-1} with a pressure of 33- 100 bar UV detection was effected at 254 nm with a sensitivity of up to 0 002 a.u.f.s The analytical column was packed with Partisil 10 ODS (25 cm × 4 5 mm I D , 10 μm particle diameter, Technicol, Stockport, U.K) protected by a guard column (2.5 cm × 4.5 mm I D) packed with Partisil 10 silica gel Normal-phase column efficiency was typically greater than 1500 plates per m for all test compounds including the internal standard

Reversed phase The mobile phase used was 12 5% dichloromethane in acetonitrile pumped at 1 ml min^{-1} A continual stream of helium passing through the solvent ensured thorough degassing UV detection was effected at 254 nm with a sensitivity of up to 0.002 a u.f s At 20 min after injection of a sample the flow-rate was increased to 3 ml min^{-1} for a further 6 min to ensure complete removal of late eluting compounds from the column

A reversed-phase column was employed for the separation (Ultrasphere ODS C_{18} with 5 μm particle diameter, 25 cm × 4.5 mm I D , Technicol) protected by a guard column (2 5 cm × 4 5 mm I D) packed with Ultrasphere ODS C_{18}, particle size 5 μm Column efficiency was greater than 3200 plates per m for the test compounds including internal standard

RESULTS

Normal-phase HPLC was employed for the separation of vitamin K_1, vitamin K_1 2,3-epoxide, and Cl-K from interfering material The retention times were 6 9 min for *cis*-vitamin K_1, 7 8 min for *trans*-vitamin K_1 and 8 7 min for vitamin K_1 2,3-epoxide The retention time for the internal standard Cl-K was 7 2 min and thus it co-eluted with vitamin K_1 Fig. 1 illustrates the trace obtained from 0.1 ml of extracted liver homogenate and indicates the time throughout which the effluent was collected.

Reversed-phase HPLC produced clear separation of vitamin K_1, vitamin K_1 2,3-epoxide and Cl-K The retention times were 14 8, 10.4 and 13.8 min,

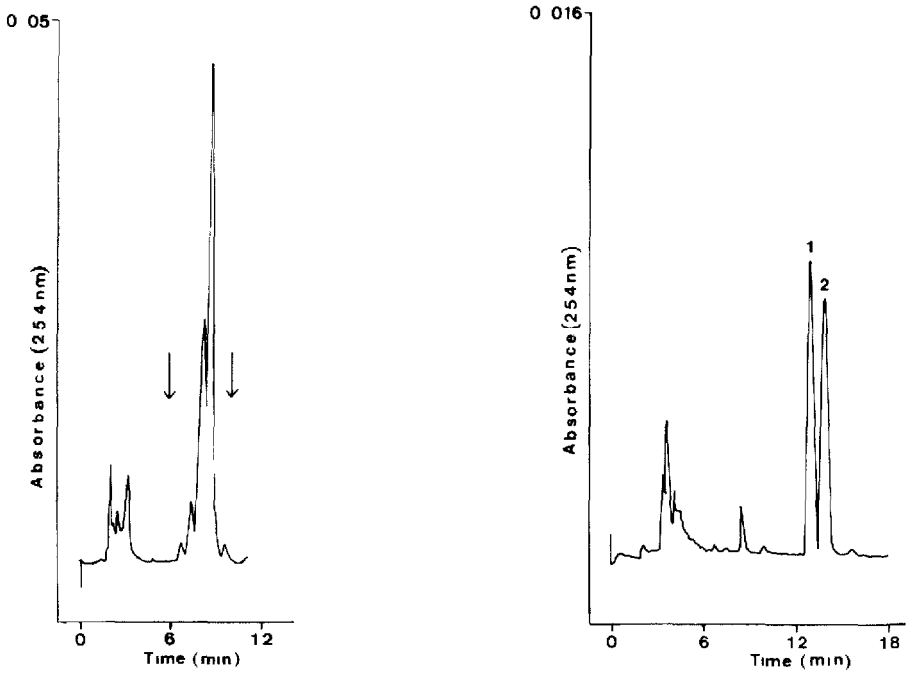

Fig 1 Normal-phase high-performance liquid chromatogram of 0 1 ml rat liver extract The arrows indicate the period throughout which eluent was collected for subsequent reversed-phase chromatography

Fig 2 Reversed-phase high-performance liquid chromatogram Extract of 33% rat liver homogenate (100 μl) obtained 3 h after intravenous administration of vitamin K_1 (1 mg kg^{-1}) Peaks 1 = Cl-K (400 ng of internal standard added), 2 = vitamin K_1

respectively. Fig 2 shows the trace obtained from the reversed-phase HPLC analysis of the fraction collected from the normal-phase HPLC system. The final calculated overall recoveries for vitamin K_1, vitamin K_1 2,3-epoxide and Cl-K from liver homogenate were 61, 77 and 50%, respectively, at a liver concentration of 0 5 g ml^{-1} The range of concentrations measured using this assay were 0 25—100 μg g^{-1} with a sensitivity of 250 ng g^{-1} Linear regression lines obtained from the standard curves were $y = 1.112x + 0\ 030$, $r = 0\ 999$ for vitamin K_1 and $y = 0\ 658x + 0\ 021$, $r = 0\ 999$ for vitamin K_1 2,3-epoxide. Intra-assay variation was calculated by the repeated chromatography of a single-spiked liver homogenate sample and gave a coefficient of variation of 1 6% Inter-assay variation was calculated by repeated chromatography of a spiked liver homogenate sample on sequential days and produced a coefficient of variation of 6 9%

Fig 3 illustrates a typical reversed-phase chromatogram obtained from the extraction of liver homogenate obtained from a rat pretreated with warfarin before receiving vitamin K_1 (1 mg kg^{-1}) Of the dose, 9 6 and 21 5% were found to be present in the liver as vitamin K_1 and vitamin K_1 2,3-epoxide, respectively A chromatogram obtained from reversed-phase analysis of the extract of liver homogenate from a rat pretreated with brodifacoum prior to administration of vitamin K_1 2,3-epoxide (1 mg kg^{-1}) is shown in Fig 4 Of

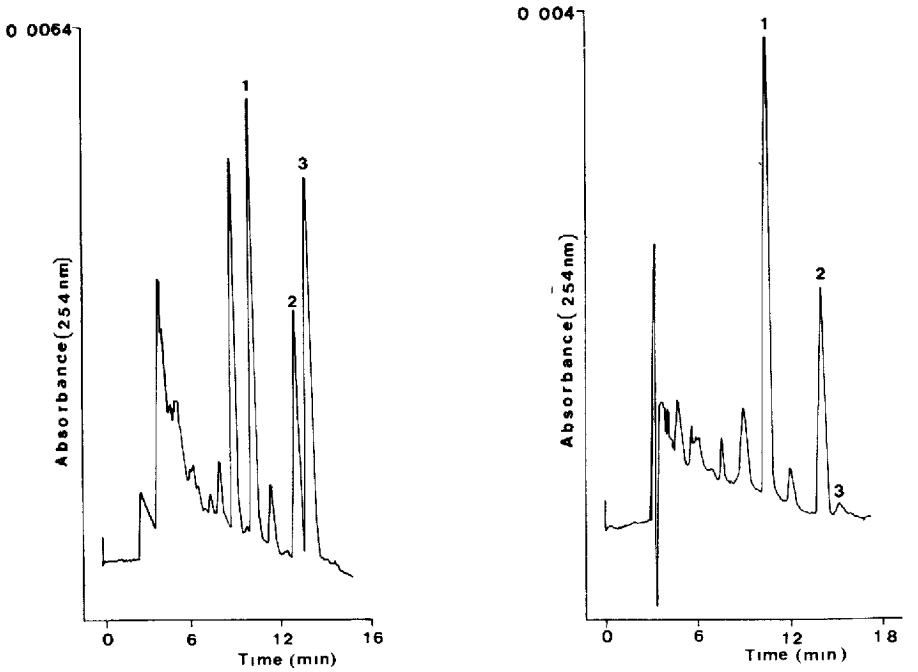

Fig 3 Reversed-phase high-performance liquid chromatogram Extract of 33% rat liver homogenate (100 μl) obtained 3 h after intravenous administration of vitamin K_1 (1 mg kg^{-1}) and 9 h after intraperitoneal administration of warfarin (63 mg kg^{-1}) Peaks 1 = vitamin K_1 2,3-epoxide, 2 = Cl-K (400 ng of internal standard added), 3 = vitamin K_1

Fig 4 Reversed-phase high-performance liquid chromatogram Extract of 33% rat liver homogenate (100 μl) obtained 3 h after intravenous administration of vitamin K_1 2,3-epoxide and 9 h after intraperitoneal administration of brodifacoum (10 mg kg^{-1}) Peaks 1 = vitamin K_1 2,3-epoxide, 2 = Cl-K (400 ng of internal standard added), 3 = vitamin K_1

TABLE I

HEPATIC *cis trans* VITAMIN K_1 RATIO IN ANTICOAGULATED RATS

Values are means of groups (n = 4) ± S D statistical significance from controls calculated using non-paired Students t-test $*p < 0\ 001$

Pretreatment	Dose (mg kg^{-1})	*cis trans* Vitamin K_1 ratio
Control		0 30 ± 0 08
Warfarin	63	2 00 ± 0 30
Brodifacoum	10	1 75 ± 0 26

the dose 76% was found to be present in the liver as vitamin K_1 2,3-epoxide and 1% as vitamin K_1.

No significant difference in the ratio of *cis trans* vitamin K_1 between warfarin and brodifacoum treated animals was found (Table I) However, the difference in the ratio of the isomers between both anticoagulated groups and the control group was significant ($p < 0\ 001$)

DISCUSSION

Recent work has led us to believe that plasma concentrations of vitamin K_1 and vitamin K_1 2,3-epoxide have only limited value in elucidating the pharmacology of vitamin K_1 and 4-hydroxycoumarin anticoagulants [8]. As the liver is the site of clotting factor synthesis it seems reasonable to suggest that better understanding would be achieved if the hepatic concentrations of vitamin K_1 and its epoxide could be determined Consequently the aim of this work was to develop an assay which would allow us to accurately measure vitamin K_1 and vitamin K_1 2,3-epoxide in the liver after intravenous administration of a pharmacological dose of either the vitamin or its epoxide.

In a recently published electrochemical detection method [12], hexane was used to extract endogenous vitamin K_1 from macerated liver However, in our hands efficient extraction of vitamin K_1 could not be achieved by hexane alone Acetone was required to extract vitamin K_1 and its epoxide from the lipophilic membranes present in liver homogenate As a result, injection of the organic extract onto either the normal-phase or reversed-phase system produced traces from which neither vitamin K_1 nor vitamin K_1 2,3-epoxide could be measured owing to interference from other lipophilic material which co-extracted Consequently sequential chromatography was employed, the first step involved a normal-phase chromatographic separation originally developed to determine plasma concentrations of vitamin K_1 and vitamin K_1 2,3-epoxide [9] Cl-K was used as internal standard instead of vitamin MK_4 (2-methyl-3-farnesylfarnesyl-1,4-naphthoquinone) as the latter co-eluted with vitamin K_1 during the subsequent reversed-phase chromatography

cis-Vitamin K_1 has far less biological activity than *trans*-vitamin K_1 in stimulating clotting factor synthesis [15], thus it was of interest to measure the relative amounts of these isomers in liver For the measurement of *cis*- and *trans*-vitamin K_1 it would have been advantageous to use the reversed-phase system as the semipreparative step and the normal-phase system for analytical purposes as it has the ability to separate the isomers. However, loss of vitamin K_1 and vitamin K_1 2,3-epoxide was experienced when a relatively large volume (8 ml) of reversed-phase eluent was evaporated, but not during evaporation of a similar volume of normal-phase eluent Therefore to measure the *cis trans* ratio, a third chromatographic step (normal phase) was required to resolve the vitamin K_1 peak (2 ml) obtained from the analytical (reversed phase) system

The apparent increase in the amount of *cis*-vitamin K_1 relative to *trans*-vitamin K_1 in the livers of those animals pretreated with either warfarin or brodifacoum can most likely be explained by a depletion of the active *trans*-vitamin K_1 due to its direct involvement in clotting factor synthesis and vitamin K_1 2,3-epoxide formation.

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