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Note

Quantitative analysis of vitamin K_1 and vitamin K_1 2,3-epoxide in plasma by electron-capture gas—liquid chromatography

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Studies on the disposition of vitamin K_1 in man and animals have so far been performed using radioactive tracer techniques [1—5]. This was necessary since specific and sensitive analytical methods for the determination of vitamin K_1 and its epoxide metabolite, including gas—liquid chromatography (GLC) [6, 7] and high-performance liquid chromatography [8], were lacking. We now describe a GLC method which permits studies on the disposition of vitamin K_1 following therapeutic doses.

EXPERIMENTAL

Reagents

All solvents were pro analysi grade (Merck, Darmstadt, G.F.R.) and were used without further purification. Vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone), vitamin $K_{2(20)}$ (menaquinone-4), and the commercially available Konakion® drops (20 mg vitamin K_1 per ml) and Marcumar® tablets (3 mg racemic phenprocoumon) were kind gifts from Hoffmann-La Roche, Grenzach-Wyhlen, G.F.R. Vitamin K_1 2,3-epoxide (2-methyl-3-phytyl-1,4-naphthoquinone 2,3-oxide) was synthesized from vitamin K_1 by the method of Tishler et al. [9].

Preparation of samples

Plasma samples of 0.2—0.8 ml were placed into 15-ml glass tubes. Ninety microlitres of an ethanolic solution of vitamin $K_{2(20)}$ (1.5 μg/ml, internal standard), 2 ml of double-distilled water, and 10 ml of *n*-hexane—absolute ethanol (1:1) were added. The tubes were fitted with PTFE-lined screw-caps and extracted for 30 min on a rotary mixer at 25 rpm. After centrifugation the upper hexane layer was removed, placed into a pointed glass tube, and evaporated to dryness under a stream of nitrogen. The residue was redissolved

in 10—25 μl of *n*-hexane and 1—2 μl were injected into the gas chromatograph. All glassware and stoppers were rinsed before use with *n*-hexane. Care was taken to protect the samples from light.

Gas–liquid chromatography

A DANI gas chromatograph (Model 3600) equipped with an electron-capture detector (radioactive source ^{63}Ni, 10 mCi, operated in the pulse mode with modulated frequency) was used. The column was a silanized O-shaped pyrex glass tube, 190 cm × 2.2 mm I.D., packed with 3% OV-17 on 90—100 mesh Anakrom Q (NEN Chemicals, Dreieichenhain, G.F.R.). Operating conditions: column oven temperature, 302°; injection port temperature, 315°; detector temperature, 305°. Oxygen-free nitrogen was used as carrier gas at a flow-rate of 80 ml/min. The column was conditioned for 24 h at 315° before use.

Calibration

Peak height ratios were calculated by dividing the height of the vitamin K_1 or vitamin K_1 2,3-epoxide peak by the height of the internal standard (vitamin $K_{2(20)}$). Standard curves of vitamin K_1 and vitamine K_1 2,3-epoxide, prepared by adding known amounts (0.005, 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4 μg) to 1 ml of pooled human plasma, were run with each set of determinations. The peak height ratios were plotted against the concentrations of vitamin K_1 and vitamin K_1 2,3-epoxide, and the least-squares regression lines were calculated. The concentrations of vitamin K_1 and vitamin K_1 2,3-epoxide in the unknown plasma samples were derived from the regression equation obtained for the standard curves.

Experiments in humans

Vitamin K_1 was administered orally in a dose of 20 mg as Konakion drops in 100 ml tap water to a healthy male subject (26 years, 75 kg). The subject fasted for 8 h before drug administration and food was not allowed until 3 h after drug administration. Three weeks after the end of this study the subject received an oral dose of 20 mg vitamin K_1. In addition phenprocoumon (0.4 mg/kg) was administered orally (Marcumar tablets) 8 h prior to the administration of vitamin K_1. Blood samples of 5 ml were drawn frequently for up to 72 h from a peripheral vein. The blood samples were heparinized and centrifuged. The plasma samples were kept frozen at —20° and protected from light.

A patient who suffered from acute pancreatitis (male, 58 years, 94 kg) received first an intravenous dose of 5 mg vitamin K_1 (0.5 ml Konakion injection solution) and 14 days later, when the acute phase of the disease was successfully treated and he was allowed to eat, an oral dose of 10 mg vitamin K_1 (Konakion drops) in about 50 ml tap water was administered.

Another patient (female, 58 years, 85 kg) who was submitted to the hospital because of signs of subarachnoid haemorrhage due to phenprocoumon overdosage (Quick value of 9% at the time of admission) received 20 mg vitamin K_1 (2 ml of Konakion, injection solution) intravenously. Eight hours later a blood sample was drawn and analyzed for vitamin K_1 and vitamin K_1 2,3-epoxide.

RESULTS AND DISCUSSION

Fig. 1 shows chromatograms obtained from blank plasma (Fig. 1A) and from plasma to which vitamin K_1, vitamin K_1 2,3-epoxide and menaquinone-4 (internal standard) were added (Fig. 1B). All three substances can be separated sufficiently under the experimental conditions described above. Retention times relative to the internal standard were 0.66 and 0.58 for vitamin K_1 and vitamin K_1 2,3-epoxide, respectively. No endogenous material present in the plasma of ten untreated subjects was found to interfere with the signal of either substance. However, a small signal with a somewhat shorter retention time than the epoxide metabolite (0.56 relative to the internal standard) was always present when aliquots from Konakion injection solutions or Konakion drops were extracted. Since this unknown material regularly amounted to 3—5% of the vitamin K_1 signal, the peak heights of vitamin K_1 2,3-epoxide could be corrected.

The calibration curves obtained for vitamin K_1 and vitamin K_1 2,3-epoxide are shown in Fig. 2. These curves were linear up to 0.4 μg/ml plasma for both compounds when 0.8 ml plasma was extracted. The lower limit of detection was 3 ng/ml plasma for both substances.

The precision of the method is shown in Table I. There was good agreement between added and recovered vitamin K_1 and vitamin K_1 2,3-epoxide at the two plasma concentrations studied. Moreover, day-to-day variations in the slopes of the calibration curves were small (coefficient of variation was below 10% within a time period of two months). The final recoveries for vitamin K_1, vitamin K_1 2,3-epoxide, and vitamin $K_{2(20)}$ (menaquinone-4) ranged from 65 to 105%. These variations, however, do not affect the accuracy of the method.

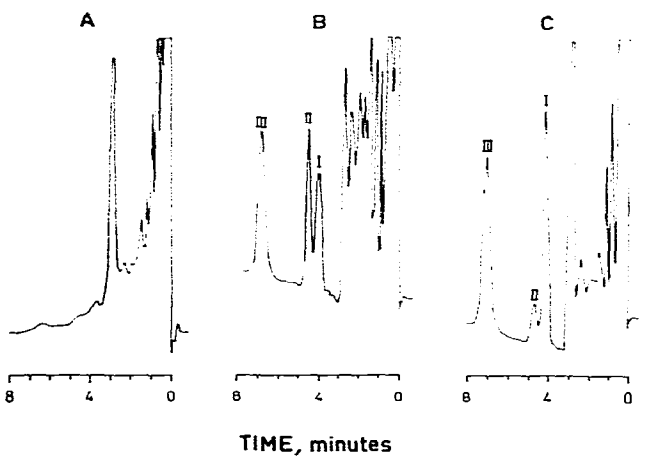


Fig. 1. Gas—liquid chromatograms of vitamin K_1 2,3-epoxide (I), vitamin K_1 (II) and vitamin $K_{2(20)}$ (III). (A) Blank extract of 0.8 ml human plasma. (B) Extract of 0.8 ml human plasma to which vitamin K_1 (0.1 μg/ml) and vitamin K_1 2,3-epoxide (0.1 μg/ml) and 0.169 μg/ml vitamin $K_{2(20)}$ were added. (C) Extract of 0.8 ml human plasma obtained from a patient who took an overdose of phenprocoumon. This patient was treated with a dose of 20 mg vitamin K_1 intravenously; 8 h later a plasma sample was obtained, spiked with vitamin $K_{2(20)}$ as internal standard and assayed.

since the ratios between the internal standard to vitamin K_1 and to vitamin K_1 2,3-epoxide remained constant (coefficient of variation below 3%, $n = 20$).

Examples of the application of the method are shown in Figs. 1, 3 and 4. The plasma concentration—time curves of vitamin K_1 and vitamin K_1 2,3-

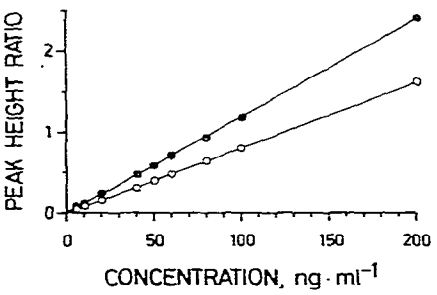
TABLE I

REPRODUCIBILITY AND ACCURACY OF THE ANALYTICAL METHOD

Concentration (μg/ml)

Vitamin K_1 added to plasma		Vitamin K_1 found		Vitamin K_1 epoxide added to plasma		Vitamin K_1 epoxide found	
1	2	1	2	3	4	3	4
0.04	0.2	0.045	0.190	0.04	0.1	0.038	0.101
0.04	0.2	0.040	0.193	0.04	0.1	0.036	0.103
0.04	0.2	0.040	0.188	0.04	0.1	0.042	0.099
0.04	0.2	0.041	0.196	0.04	0.1	0.038	0.101
Mean ± S.D.							
0.04	0.2	0.042 ± 0.002	0.192 ± 0.004	0.04	0.1	0.039 ± 0.003	0.101 ± 0.001

epoxide in the healthy male subject who received an oral dose of 20 mg vitamin K_1 8 h after an oral dose of 30 mg phenprocoumon are shown in Fig. 3. After the 20-mg oral dose plasma levels of the vitamin rose sharply at about 3 h after administration and peak plasma levels were reached at about 4 h. Thereafter, plasma levels declined at least bi-exponentially with time. The shape of the curve is similar to those obtained with [^{3}H]vitamin K_1 [1]. Moreover, the



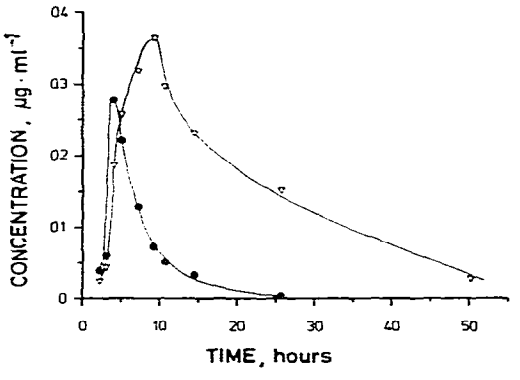


Fig. 2. Calibration graphs for vitamin K_1 (●) and vitamin K_1 2,3-epoxide (○) obtained from human plasma to which different concentrations of the compounds were added. Samples of 0.8 ml plasma were assayed. Peak height ratio = height of vitamin K_1 (or vitamin K_1 2,3-epoxide)/height of vitamin $K_{2(20)}$.

Fig. 3. Plasma concentration—time curves of vitamin K_1 (●) and vitamin K_1 2,3-epoxide (▽) in a healthy male subject who received a single oral dose of 20 mg vitamin K_1 8 h after a single oral dose of 30 mg phenprocoumon.

epoxide metabolite accumulates in the plasma and its concentrations even exceeded those of the native vitamin. This metabolite could not be detected in the study performed three weeks earlier where this subject received the same oral dose of vitamin K_1 only. In this study the magnitude of the area under the plasma concentration—time curve of vitamin K_1 was similar to that shown in Fig. 3. The accumulation of the epoxide metabolite in the plasma following pretreatment with phenprocoumon most likely reflects interruption of the vitamin K-epoxide cycle due to inhibition of the epoxide reductase by phenprocoumon [10, 11].

The plasma concentration—time curve of vitamin K_1 following intravenous (5 mg) and oral (10 mg) administration of the vitamin in the patient suffering from pancreatitis is shown in Fig. 4. Obviously, the systemic availability of vitamin K_1 following oral administration was very small (about 15%) when dose-corrected areas were used. This probably reflects an impaired absorption of the vitamin due to the underlying disease [1].

No quantifiable amounts of vitamin K_1 2,3-epoxide could be detected in our studies following administration of single oral and intravenous doses of vitamin K_1. However, in the presence of phenprocoumon large amounts of the epoxide metabolite appeared in the plasma. This was seen in the healthy subject treated additionally with phenprocoumon (Fig. 3) and also in the patient intoxicated with phenprocoumon (see Fig. 1C).

Vitamin $K_{2(20)}$ (menaquinone-4), which was used as the internal standard in our method, was not detected in the plasma of untreated, vitamin K_1 treated, and vitamin K_1 and phenprocoumon treated subjects.

The analytical method described may overcome some of the limitations connected with the use of radioactive material. The sensitivity of this method is sufficient to detect with accuracy vitamin K_1 plasma concentrations produced

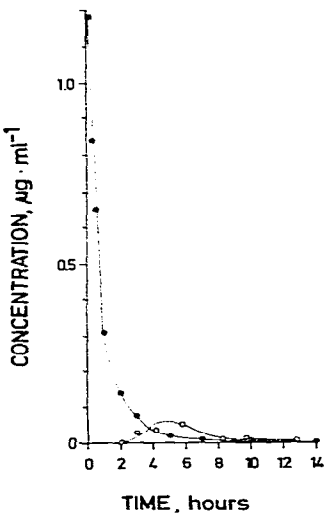


Fig. 4. Plasma concentration—time curves of vitamin K_1 following an intravenous dose of 5 mg (•) and following an oral dose of 10 mg (○) vitamin K_1 to a subject who suffered from pancreatitis.

by therapeutic doses of this vitamin. Moreover, it is possible to follow in the plasma the formation of vitamin K_1 2,3-epoxide in anticoagulant treated subjects, thus permitting studies in man on the effect of these drugs on vitamin K_1 metabolism.

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REFERENCES

1 M.J. Shearer, A. McBurney and P. Barkhan, Vitam. Horm., 32 (1974) 513.
2 M.J. Shearer, A. McBurney, A.M. Breckenridge and P. Barkhan, Clin. Sci. Mol. Med., 52 (1977) 621.
3 J.T. Matschiner, R.G. Bell, J.M. Amelotti and T.E. Knauer, Biochim. Biophys. Acta, 201 (1970) 309.
4 P.T. Caldwell, P. Ren and R.G. Bell, Biochem. Pharmacol., 23 (1974) 3353.
5 T.D. Bjornsson, S.E. Swezey, P.J. Meffin and T.F. Blaschke, Thromb. Haemostas., 39 (1978) 466.
6 G.H. Dialameh and R.E. Olson, Anal. Biochem., 32 (1969) 263.
7 A.J. Sheppard, A.R. Prosser and W.D. Hubbard, J. Amer. Oil Chem. Soc., 49 (1972) 619.
8 G.R. Ellioth, E.M. Odam and M.G. Townsend, Biochem. Soc. Trans., 4 (1976) 615.
9 M. Tishler, L.F. Fieser and N.L. Wendler, J. Amer. Chem. Soc., 62 (1940) 2866.
10 D.S. Whitlon, J.A. Sadowski and J.W. Suttie, Biochemistry, 17 (1978) 1371.
11 R.G. Bell, Fed. Proc., 37 (1978) 2599.