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Note

Improved method for quantitative analysis of vitamin K_1 and vitamin K_1 2,3-epoxide in human plasma by electron-capture gas—liquid capillary chromatography

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Application of capillary columns in gas chromatography results in higher resolution, shorter analysis time and higher sensitivity compared to packed columns [1]. In recent years high-temperature-resistant stationary phases for capillaries have been developed and the introduction of fused-silica capillaries simplified installation and handling of such columns. We therefore tested if the use of a fused-silica capillary column instead of a packed column results in an improvement of our recently published gas-chromatographic method for the quantitative determination of vitamin K_1 and vitamin K_1 2,3-epoxide in plasma of man [2].

EXPERIMENTAL

Reagents

All solvents were pro analysi grade (Merck, Darmstadt, F.R.G.) and were used without further purification. *Cis*- and *trans*-vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone), racemic vitamin $K_{2(20)}$ (menaquinone-4), *cis*- and *trans*-vitamin K_1 2,3-epoxide (2-methyl-3-phytyl-1,4-naphthoquinone 2,3-oxide), commercially available Konakion® solution (1 ml ampoules containing 10 mg of vitamin K_1) were kind gifts from Dr. Weber and Dr. Gloor (Hoffmann-La Roche, Basel, Switzerland).

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Gas—liquid chromatography

A Dani gas chromatograph (Model 3900) 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 25 m × 0.32 mm I.D. fused-silica capillary column with chemically bonded stationary phase (CP^{tm} Sil 5 CB, film thickness 0.12 μm; Chrompack Nederland B.V., Middelburg, The Netherlands). A Dani splitter introduction device was used. Injections of diluted samples (0.8—1.5 μl of *n*-heptane) were made by the Grob [3] splitless injection technique into the closed injection port at 280°C while the column was maintained at 60—80°C. After 40 s the injector was flushed with carrier gas (split ratio 1:50) and the column oven was quickly heated up to its final temperature of 285°C (heating rate about 40°C/min). The detector was set at 330°C. Oxygen-free helium was used as carrier gas (inlet pressure 1.2 bar) and nitrogen as make-up gas for the detector (flow-rate 30 ml/min).

Calibration

Calibration curves were constructed by adding known amounts of vitamin K_1 (Konakion ampoules) and *trans*-vitamin K_1 2,3-epoxide (12.5, 25, 50, 100, 150, 200 ng/ml) to pooled human plasma. The peak height ratios of vitamin K_1 and *trans*-vitamin K_1 2,3-epoxide to the internal standard (vitamin $K_{2(20)}$) were plotted against the concentrations of vitamin K_1 and *trans*-vitamin K_1 2,3-epoxide. Corrections had been performed which accounted for the fact that *trans*-vitamin K_1 2,3-epoxide contained about 10% of *cis*-vitamin K_1 2,3-epoxide and the Konakion (vitamin K_1) about 5% of *trans*-vitamin K_1 2,3-epoxide as impurities (as checked by gas chromatography).

The least-squares regression lines for vitamin K_1 and *trans*-vitamin K_1 2,3-epoxide were fitted through the data points. The vitamin K_1 and *trans*-vitamin K_1 2,3-epoxide concentrations of unknown plasma samples were determined by using the regression equations of the calibration curves which were run with each set of determinations of unknown plasma samples. Samples containing concentrations higher than 200 ng/ml for vitamin K_1 or *trans*-vitamin K_1 2,3-epoxide were suitably diluted with blank plasma.

Preparation of samples

Plasma samples of 0.4 ml were placed into 15-ml glass tubes. A volume of 50 μl of an ethanolic solution of vitamin $K_{2(20)}$ (2.0 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 *n*-hexane layer was removed, placed into a pointed glass tube, and evaporated to dryness under a stream of nitrogen. The residue was redissolved in 40 μl of *n*-heptane and 0.8—1.5 μl were injected into the gas chromatography by the Grob [3] splitless injection technique.

RESULTS AND DISCUSSION

Fig. 1 shows a chromatogram obtained after splitless injection of 2 ng of *cis*- and *trans*-vitamin K_1 2,3-epoxide, vitamin K_1 (Konakion) and vitamin

$K_{2(20)}$. There was a baseline separation between all four compounds. *Cis*- and *trans*-isomers of vitamin K_1 could not be separated under our experimental conditions. The retention time of the vitamin $K_{2(20)}$ peak, which was eluted last, was about 12 min. Since cooling down of the oven from 285°C to injection temperature of 80°C takes about 6—8 min, repeated injections could be performed at 20—30-min intervals. The vitamin $K_{2(20)}$ peak showed a small "shoulder" at the end of the peak (Fig. 1,★). This was probably due to an unknown impurity or to an incomplete separation of a minor amount of the *cis*- or *trans*-isomer of vitamin $K_{2(20)}$. Since the isomers of vitamin $K_{2(20)}$ were not available this phenomenon could not be checked. When the *trans*-vitamin K_1 2,3-epoxide was injected it contained about 10% of its *cis*-isomer. The commercial available Konakion solution consisted of about 95% of vitamin K_1 and 5% of *trans*-vitamin K_1 2,3-epoxide.

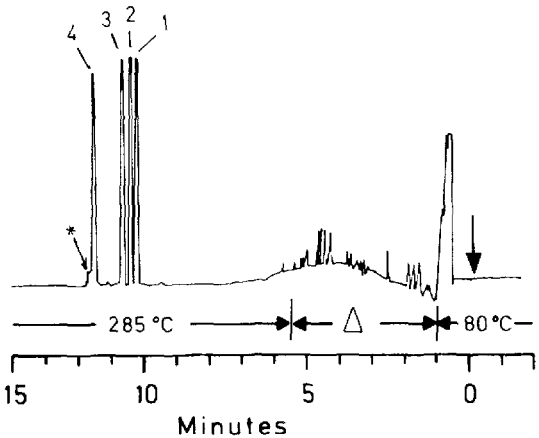

Fig. 1. Chromatograms after splitless injection of 1 µl of *n*-heptane containing *trans*- and *cis*-vitamin K_1 2,3-epoxide, racemic vitamin K_1 and racemic vitamin $K_{2(20)}$ (menaquinone 4), 2 ng of each. Peak 1 = *trans*-vitamin K_1 2,3-epoxide; peak 2 = *cis*-vitamin K_1 2,3-epoxide; peak 3 = racemic vitamin K_1; peak 4 = racemic vitamin $K_{2(20)}$ (* = resolution of an impurity which could be the *cis*- or *trans*-isomer of vitamin $K_{2(20)}$. Injection (↓) was done at an oven temperature of 80°C with the closed splitter. The splitter was opened after 40 s (split ratio 1:50) and the oven was heated up at maximal heating rate (△) to final temperature of 285°C. This temperature was reached at about 5—6 min after injection.

Examples of chromatograms from plasma extracts of blank plasma and plasma to which 15 ng/ml vitamin K_1 and 15 ng/ml *trans*-vitamin K_1 2,3-epoxide were added are shown in Fig. 2A. The *cis*-vitamin K_1 epoxide peak which resulted from an impurity of the added *trans*-vitamin K_1 2,3-epoxide appeared even visible between the K_1 and K_1 epoxide peak in the chromatogram (★ in Fig. 2A). No endogenous material in the blank plasma of ten healthy subjects and patients interfered with either vitamin K_1, *cis*- or *trans*-vitamin K_1 epoxide or vitamin $K_{2(20)}$ signals (Fig. 2B).

The calibration curves for vitamin K_1 and *trans*-vitamin K_1 2,3-epoxide were linear up to concentrations of 200 ng/ml when 0.4-ml plasma samples were extracted, and passed through the origin ($r > 0.98$; intercept at the y-axis < 0.01). The lower limit of quantitative detection of vitamin K_1 and vitamin K_1 2,3-epoxide was about 5 ng/ml in plasma. This limit of detection in plasma was due to the fact that plasma extracts must be diluted by a factor of 1:40

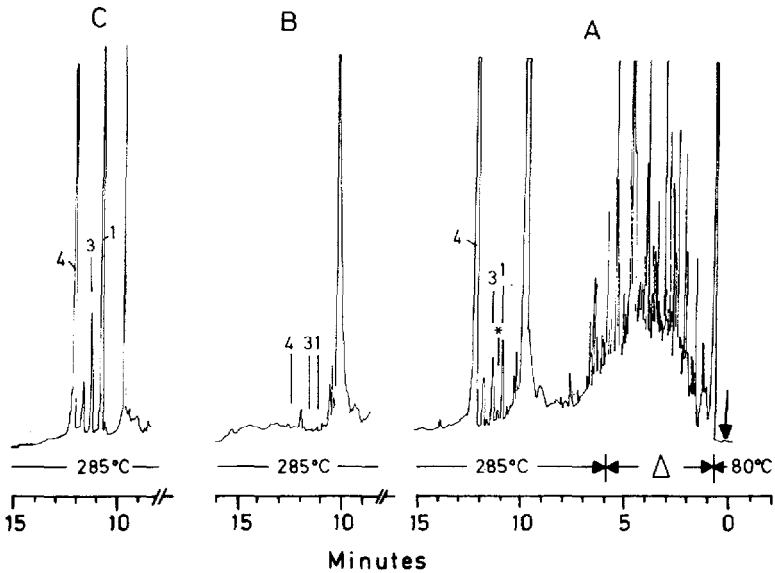

Fig. 2. Chromatograms of vitamin K_1 (peak 3), *trans*-vitamin K_1 2,3-epoxide (peak 1) and vitamin $K_{2(20)}$ (peak 4) from plasma extracts. (A) Extract of 0.4 ml of human plasma to which vitamin K_1 (15 ng/ml) and *trans*-vitamin K_1 2,3-epoxide (15 ng/ml) and vitamin $K_{2(20)}$ (250 ng/ml) were added. (B) Blank extract of 0.4 ml of human plasma. (C) Extract of 0.4 ml of plasma from a patient who was under treatment with the oral anticoagulant drug phenprocoumon (plasma concentration of 0.64 μg/ml). This patient had received a single intravenous dose of 10 mg of vitamin K_1; 4 h later a plasma sample was drawn, spiked with vitamin $K_{2(20)}$ as internal standard and analyzed. * indicates the even visible *cis*-vitamin K_1 2,3-epoxide peak (about 1.5 ng/ml) since the administered *trans*-vitamin K_1 2,3-epoxide contained about 10% of its *cis*-isomer (A). Δ = heating period, heating rate of about 40°C/min.

to avoid an overload of the column by other coextracted lipids. For pure substances the lower limit of detection was about 10—20 pg.

The accuracy and reproducibility of the method is given in Table I. There was a good agreement between added and found vitamin K_1 and vitamin K_1 epoxide at the two plasma concentrations studied. Moreover, day-to-day variations in the slopes of the calibration curves were small (coefficient of variation below 10% within a time period of 3 months).

An example of the application of the method is shown in Fig. 2C. From a subject treated with the anticoagulant drug phenprocoumon (phenprocoumon plasma concentration of 0.64 μg/ml) and who received in addition 10 mg of vitamin K_1 intravenously, a plasma sample was drawn 4 h after injection of the vitamin. The concentration of vitamin K_1 in this sample was 78 ng/ml and that of vitamin K_1 2,3-epoxide 211 ng/ml. In former studies it was shown that in subjects treated with oral anticoagulant drugs the plasma levels of vitamin K_1 2,3-epoxide were maximal at 3—4 h after administration of vitamin K_1 [4]. Interestingly, the endogenously formed vitamin K_1 2,3-epoxide was the *trans*-isomer as shown in Fig. 2C.

Thus, the described capillary gas chromatographic method is suitable to study the kinetics and the metabolism of vitamin K_1 and vitamin K_1 2,3-epoxide in plasma of man following therapeutic doses of these vitamins. The lower limit of detection for vitamin K_1 and vitamin K_1 2,3-epoxide in plasma

TABLE I

REPRODUCIBILITY AND ACCURACY OF THE ANALYTICAL METHOD

	Concentration (ng/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	1	2	1	2
	25.0	150.0	27.3	143.8	25.0	150.0	27.4	152.4
	25.0	150.0	22.3	165.3	25.0	150.0	19.7	151.2
	25.0	150.0	29.8	154.9	25.0	150.0	30.6	151.1
	25.0	150.0	25.6	154.5	25.0	150.0	23.2	153.5
	25.0	—	25.4	—	25.0	—	24.0	—
	25.0	—	30.4	—	25.0	—	28.2	—
	25.0	—	22.5	—	25.0	—	29.5	—
Mean ± S.D.	25.0	150.0	26.2 ±3.2	154.6 ±8.7	25.0	150.0	26.1 ±3.9	152.1 ±1.2
Coefficient of variation (%)			12	6			15	1

was about the same as that obtained with packed columns [2]. However, there are some profound advantages using the silica capillary column over the packed column:

(1) There was a complete baseline separation between vitamin K_1 and the isomers of vitamin K_1 2,3-epoxide. Using the packed column isomers of vitamin K_1 2,3-epoxide could not be separated and separation between vitamin K_1 and vitamin K_1 epoxide was incomplete.

(2) Efficiency and resolution remained constant over months even after repeated daily injections of plasma extracts. Using the packed column peak broadening and loss of efficiency often occurred when plasma extracts were injected repeatedly.

(3) Replacing the capillary column by a new one from the same manufacturer gives identical resolution and retention times. Using the packed column efficiency was influenced by minor differences in manufacturing of the glass column, and by packing and conditioning procedures.

(4) The high sensitivity of the method for pure substances should allow the detection of endogenous vitamin K_1 plasma levels, following adequate prepurification of plasma samples.

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