papers and notes on methodology

Application of high-performance liquid chromatography to assay phylloquinone (vitamin K_1) in rat liver

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Abstract A specific and sensitive method based on high-performance liquid chromatography to measure vitamin K_1 in rat liver is described. The procedure consists of three chromatographic steps, a preliminary purification of lipid extracts on Sep-Pak silica columns, a preparative stage using adsorption HPLC, and a final reversed-phase HPLC stage in which vitamin K_1 is resolved from remaining contaminants and quantified by reference to an internal standard (vitamin K_1 2,3-epoxide). The "within-run" coefficient of variation of the assay for vitamin K_1 in 12 livers was 11.1% (n = 24). The concentration of vitamin K_1 in liver of male rats fed a control diet containing no alfalfa was 8.0 ng/g (wet weight of liver) while female rat liver gave a mean value of 7.3 ng/g (wet weight of liver). Liver from male rats fed chow containing alfalfa gave a mean value of 44 ng K_1/g (wet weight of liver).—**Haroon, Y., and P. V. Hauschka.** Application of high-performance liquid chromatography to assay phylloquinone (vitamin K_1) in rat liver. *J. Lipid Res.* 1983. **24:** 481–484.

Supplementary key word vitamin K

Previous quantitative measurements of the very low concentrations of K vitamins in animal livers have employed multidimensional classical chromatographic techniques such as adsorption (1), reversed-phase (1, 2), and argentation chromatography (2). Such methods have not always proved reliable owing to the limitations of sensitivity and the difficulty in obtaining pure fractions of K vitamins for their quantitation by ultraviolet spectroscopy. In addition, prohibitively large quantities of starting material (1–5 Kg) are required to obtain sufficient K vitamins for their identification and measurement (3, 4).

The potential usefulness of high-performance liquid chromatography (HPLC) for the quantitation of vitamin K_1 (phylloquinone) in foods has recently been demonstrated by Shearer and co-workers (5, 6) and also by Thompson, Hatina, and Maxwell (7). The present report is concerned with the application of previously developed HPLC methodology to assay phylloquinone in rat liver.

MATERIALS AND METHODS

Chemicals

Phylloquinone 2,3-epoxide (K_1 epoxide) was synthesized from phylloquinone (Sigma, St. Louis, MO) as described previously (8). 2-Chloro-phylloquinone (chloro-K_1) was a gift (Drs. J. A. Sadowski and J. W. Suttie, Department of Biochemistry, University of Wisconsin, Madison, WI), and K_1(I-H_2) was generously supplied by Drs. Brubacher and Weber (F. Hoffman-LaRoche & Co., CH-4002, Basle, Switzerland).

Solvents were HPLC grade (Burdick and Jackson, Muskegan, MI), and 50% water-saturated dichloromethane was prepared as previously described (8).

Animals

Sprague-Dawley CD strain rats (Charles River Breeding Laboratories, Inc., Wilmington, MA) were fed chow

Abbreviations: HPLC, high-performance liquid chromatography; Reference to specific forms of vitamin K is made in accordance with the recommendation of the IUPAC-IUB Commission on Biochemical Nomenclature (1965. *Biochem. Biophys. Acta.* **107:** 5). The K_2 vitamins are menaquinones, abbreviated MK, and vitamin K_1 is phylloquinone, abbreviated K_1. Vitamin K_1 with the 2′,3′ double bond hydrogenated is represented as dihydro-K_1 and abbreviated to K_1(I-H_2).

(Ralston Purina Company) that was supplemented by the manufacturer with alfalfa (5001) or chow unsupplemented with alfalfa (5006). The rats were killed when they were 7–8 weeks old and weighed 125–200 g. Livers were removed and stored in the dark at −20°C until analysis.

High-performance liquid chromatography

The liquid chromatographs were equipped with reciprocating pumps (model 6000A, Waters Assoc., Milford, MA), a variable-wavelength ultraviolet detector (model 450, Waters Assoc.) and an automated injection system (model 710A, Waters Assoc.). Adsorption HPLC was performed on a column of Partisil-5 silica (Whatman, Inc., Clifton, NJ); for reversed-phase HPLC either a Zorbax-ODS (Dupont, Wilmington, DE) or a Hypersil-ODS (Shandon Southern Products, Ltd., Sewickley, PA) column was used. All columns were purchased prepacked from the suppliers and were 250 mm length and 4.6 mm internal diameter. Columns were operated at a flow rate of 1.0 ml/min and the detection wavelength for both HPLC steps in the assay of K_1 was 270 nm.

Lipid extraction

Whole livers weighing 7–10 g were ground with anhydrous sodium sulfate in a mortar. The macerated tissue was transferred with hexane (50 ml) into a 100-ml conical flask and shaken mechanically for 2 min. After addition of the internal standard (K_1 epoxide) the lipid extract was filtered through a glass microfiber filter (Whatman, GF/A) in a Buchner funnel. The filtrate was divided into two equal portions and dried over activated sodium sulfate. The solvent was removed under N_2 in a water bath at 60°C.

Chromatography of lipid extracts

Details of the fractionation scheme employed for the purification of K_1 from crude lipid extracts have already been published (5, 6). In addition, in these studies the conventional chromatographic step previously performed on slurry packed silica gel (Kieselgel 60) columns was replaced by prepacked Sep-Pak columns (Waters Assoc.). The lipid extract was dissolved in hexane and 50–100 mg was applied to columns by means of a 10-ml syringe fitted with a Luer tip. Hydrocarbons were first removed by eluting with hexane (8 ml) and a vitamin K fraction that included the internal standard and other lipids (i.e., triglycerides) was obtained by eluting with 3% diethylether in hexane (8 ml). After removal of solvents, "the vitamin K-containing fraction" was redissolved in hexane (200 μl), and two to three injections of 50 μl each were applied to a column of Partisil-5 silica. The fraction of the eluent corresponding to K_1 and internal standard was collected (6) and dried over a stream of N_2 in a water bath at 60°C. These fractions were further subjected to reversed-phase HPLC as described previously (6).

Quantitation of K_1 in liver samples was achieved by the peak height ratio (K_1/K_1 epoxide) method already published (6).

RESULTS AND DISCUSSION

Extraction of phylloquinone (K_1)

Table 1 shows the solvents and conditions of extraction that were studied in order to develop a suitable and efficient method for extracting K_1 from rat liver. It was observed that liver samples that had been freeze-dried contained significantly less K_1 compared to tissue that had been ground with anhydrous sodium sulphate before extraction (Table 1). This difference was attributed to the volatility of K_1 under conditions of freeze-drying. Matschiner, Taggart, and Amelotti (1) also reported a loss of vitamin K from beef liver after freeze-drying.

Although acetone, hexane, and chloroform–methanol 2:1 were equally effective in extracting K_1 from rat liver (Table 1), hexane was chosen as the extracting solvent for the assay of K_1 because it allowed extraction of a nonpolar "vitamin K-containing fraction" while reducing contamination by polar lipids.

Chromatography of lipid extracts

A modification of the three-stage chromatographic method previously described was used to assay K_1 in liver samples (6). The first step was a preliminary purification by conventional liquid chromatography employing Sep-Pak silica cartridges. In this step a "vitamin K-containing fraction" was eluted early from the column thus providing a rapid fractionation from the bulk of interfering lipids that were more polar than vitamin K and were retained by the silica. The removal of these polar lipids was necessary to prevent their deposition on the microparticulate silica columns used later.

In the second chromatographic stage, the nonpolar fraction was purified further by preparative HPLC on

TABLE 1. Conditions for extraction of vitamin K_1 from rat liver

Solvent	Cell Disruption	Wet Weight[a]
		ng/g
Acetone	macerated	19.7
Chloroform–methanol 2:1	macerated	19.2
Hexane	macerated	18.3
Hexane	freeze-dried	10.4

[a] Three livers were pooled and duplicate aliquots of 5 g each were extracted.

a column of Partisil-5. The sample was injected on the HPLC column and the fraction of the eluent that corresponded to K_1 and internal standard was collected as described previously (6).

The final stage of the assay involved the resolution of K_1 and internal standard from remaining impurities on columns of either Zorbax-ODS or Hypersil-ODS (6).

Representative chromatograms of the adsorption and reversed-phase stages of the assay for K_1 in rat liver are shown in **Fig. 1a** and Fig. 1b, respectively.

Choice of internal standard

Because the assay required multiple chromatographic steps, it was essential for accurate quantitation to correct for losses of the vitamin during the procedure. It was considered that the simplest and most accurate method would be to find a suitable compound that could be detected photometrically so that calibration and quantitation could be achieved by peak:height ratios. To date, we have evaluated the compounds chloro-K_1, K_1 epoxide, and dihydro-K_1 as internal standards. Adsorption HPLC resolves chloro-K_1 and dihydro-K_1 from K_1 but not K_1 and K_1 epoxide, and because it was critical to collect the least possible volume of eluent containing K_1 and internal standard from the preparative stage of the assay, K_1 epoxide was chosen as the internal standard. Another reason why chloro-K_1 and dihydro-K_1 were unsuitable as internal standards was the presence of other unidentified peaks from liver co-eluting with these compounds on the final reversed-phase stage of the assay.

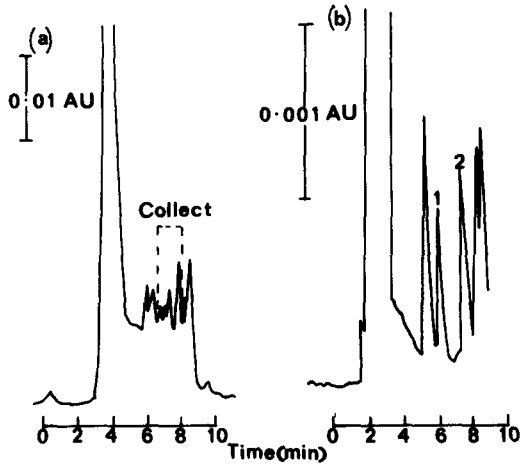

Fig. 1. Chromatograms of the two stages of HPLC in the assay of K_1 in rat liver. (a) Semi-preparative stage on Partisil-5 with a mobile phase of hexane–50% water-saturated dichloromethane 8:2 (v/v) and UV detection at 270 nm. (b) Analytical reversed-phase stage on Zorbax-ODS and a mobile phase of methanol–dichloromethane 3:1 (v/v) and UV detection at 270 nm. Peaks: 1 = K_1 epoxide (internal standard); 2 = K_1.

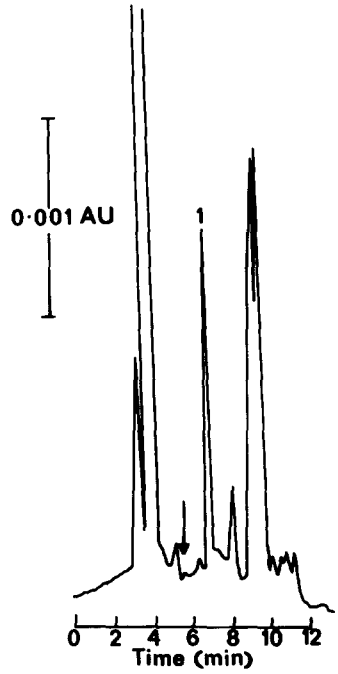

Fig. 2. Reversed-phase chromatogram showing the retention volume (indicated by an arrow) of endogenous K_1 epoxide in rat liver. An equivalent of 2 g of liver was injected. The chromatographic system was the same as for Fig. 1(b).

Although one objection of K_1 epoxide as an internal standard is its known occurrence as a metabolite in rat liver (9), in practice this limitation did not significantly affect the analysis because at the wavelength (270 nm) of detection used in the assay for K_1, endogenous K_1 epoxide could not be detected in liver (**Fig. 2**).

Precision and accuracy of HPLC assay

The accuracy of the method was assessed by adding known amounts of K_1 to liver samples and measuring K_1 content by the assay procedure. After subtracting the endogenous levels of K_1 in the sample, assayed separately, good agreement was found between the amounts of K_1 added and those calculated (**Table 2**). The "within-run" coefficient of variation for the assay of vitamin K_1 in 12 livers was 11.1% (n = 24).

TABLE 2. Accuracy of HPLC assay

Concentration	
K_1 Added to Liver	K_1 Recovered in Liver[a]
ng/g	
5.0	6.9
10.0	9.2
15.0	16.8
20.0	18.7
25.0	27.1

[a] Values are mean of duplicate analyses and were calculated by subtracting the endogenous level of K_1 determined in a separate assay.

K_1 content of rat liver

The concentration of K_1 in liver of six male rats fed a diet containing no alfalfa was 8.0 ng/g ± 2.7 SD (range 4.9–12.2 ng/g). This was not statistically different from the amount of K_1 in liver from six female rats, which gave a mean value of 7.3 ng/g ± 2.7 SD and a range of 3.0–11.1 ng/g. The mean hepatic concentration of K_1 in male rats fed Purina chow containing alfalfa (source of K_1) was found to be 44 ng/g ± 1.5 SD (n = 4). These findings illustrate that K_1 found in rat liver is derived mainly from nutritional sources.

Although our values for K_1 in rat liver are considerably lower than those reported by Matschiner and Doisy (10), who found that female rat liver contained 100 ng/g (phylloquinone equivalent) while livers from male rats contained about 30% less, it is important to realize that their values were obtained by the chick bioassay. Bioassay measures the total vitamin K (K_1 and MKs) content while the HPLC assay measures only K_1. Therefore, it is likely that the higher bioassay values reflect the presence of menaquinones that would not be detected by the HPLC assay for K_1. In this respect, we have so far been unable to obtain from the preparative stage of the assay sufficiently pure fractions of MKs for their detection and quantitation.

Another reason for the discrepancy between the HPLC and bioassay data may be related to the biological activity differences between K_1 and MKs. For example, Matschiner and Taggart (11) have shown that on a molar basis MKs −7, −9, and −10 are considerably more active than K_1 when administered intracardially to the rat. Since the bioassay was standardized by relating prothrombin times to concentrations of K_1, it is possible to obtain the same degree of prothrombin activity as that given by K_1 with much lower concentrations of MKs.

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REFERENCES

1. Matschiner, J. T., W. V. Taggart, and J. M. Amelotti. 1967. The vitamin K content of beef liver: detection of a new form of vitamin K. *Biochemistry.* **6:** 1243–1248.
2. Matschiner, J. T., and M. J. Amelotti. 1968. Characterization of vitamin K from bovine liver. *J. Lipid Res.* **9:** 176–179.
3. Duello, T. J., and J. T. Matschiner. 1972. Characterization of vitamin K from human liver. *J. Nutr.* **102:** 331–336.
4. Rietz, P., U. Gloor, and O. Wiss. 1970. Menachinone aus menschlicher leber und faulschlamm. *Int. Z. Vitamforsch.* **40:** 351–362.
5. Shearer, M. J., V. Allan, Y. Haroon, and P. Barkhan. 1980. Nutritional aspects of vitamin K in the human. *In* Vitamin K Metabolism and Vitamin K-dependent Proteins. J. W. Suttie, editor. University Park Press, Baltimore, MD. 317–327.
6. Haroon, Y., M. J. Shearer, S. Rahim, W. G. Gunn, G. McEnery, and P. Barkhan. 1982. The content of phylloquinone (vitamin K_1) in human milk, cows' milk and infant formula foods determined by high-performance liquid chromatography. *J. Nutr.* **112:** 1105–1117.
7. Thompson, J. N., G. Hatina, and W. B. Maxwell. 1979. Determination of vitamins E and K in foods and tissues using high-performance liquid chromatography. *In* National Bureau of Standards Special Publication Trace Organic Analysis. Proc. 9th Materials Research Symp. 279–281.
8. Haroon, Y., M. J. Shearer, and P. Barkhan. 1980. Resolution of phylloquinone (vitamin K_1), phylloquinone 2,3-epoxide, 2-chloro-phylloquinone and their geometric isomers by high-performance liquid chromatography. *J. Chromatogr.* **200:** 293–299.
9. Matschiner, J. T., R. G. Bell, J. M. Amelotti, and T. E. Knauer. 1970. Isolation and characterization of a new metabolite of phylloquinone in the rat. *Biochim. Biophys. Acta.* **201:** 309–315.
10. Matschiner, J. T., and E. A. Doisy, Jr. 1966. Bioassay of vitamin K in chicks. *J. Nutr.* **90:** 97–100.
11. Matschiner, J. T., and W. V. Taggart. 1968. Bioassay of vitamin K by intracardial injection in deficient adult male rats. *J. Nutr.* **94:** 57–59.