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REACTION OF VITAMIN K AND DITHIOTHREITOL ON REVERSED-PHASE C_{18} HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC COLUMNS

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SUMMARY

A non-enzymatically catalyzed side reaction between dithiothreitol and vitamin K occurs on reversed-phase C_{18} high-performance liquid chromatographic columns. Excess dithiothreitol may be effectively removed from enzymatic reaction mixtures with N-ethylmaleimide. A low polarity gradient elution system is reported which gives quantitative recoveries (greater than 95%) of the vitamins in a concentration range useful for analysis of enzymatically catalyzed reactions.

INTRODUCTION

Vitamin K-dependent enzymes are stabilized by thiols[1], and therefore are assayed routinely in concentrations of dithiothreitol as high as 10 m*M*, much of which can be extracted into organic solvents. However, vitamin K and its metabolites readily react with thiols[2,3]. In attempting to quantitate vitamin K and its enzymatic metabolites using high-performance liquid chromatographic (HPLC) methods[4], we have observed side reactions of vitamin K with dithiothreitol on C_{18} HPLC columns. If small amounts of vitamin K are retained on the column, after repeated injections over a period of weeks, significant amounts can accumulate in the solid phase. When solutions containing dithiothreitol and vitamin K are then injected, reactions sporadically occur on the column which seriously interfere with quantitation of the vitamin K-dependent enzymes. We report here a low polarity gradient elution procedure in which quantitative elution of vitamin K and its metabolites is achieved and a method for removing dithiothreitol from completed enzymatic reaction mixtures prior to injection of vitamins on the HPLC column.

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MATERIALS AND METHODS

Chemicals

Renex 30 was obtained from ICI (Wilmington, DE, U.S.A.) and β-D-glucopyranoside from Calbiochem-Behring (La Jolla, CA, U.S.A.). Protein assay reagents were from Bio Rad Labs. (Richmond, CA, U.S.A.). Warfarin, 5,5′-dithiobis-2-nitrobenzoic acid, phenylmethylsulfonyl fluoride, Vitamin K (2-methyl-3-phytyl-1,4-naphthoquinone) and peptide substrate (Phe-Leu-Glu-Glu-Ile) were from Sigma (St. Louis, MO, U.S.A.). HPLC grade phosphoric acid (85%) was from Fisher (Fairlawn, NJ, U.S.A.). All other solvents were HPLC grade from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

Extraction of vitamin K and its metabolites

Quantitation of vitamins, preparation of enzymes and enzymatic assays for vitamin K epoxidase have been described previously[5]. Epoxide reductase reaction mixtures contained 5–10 mg ml^{-1} microsomal protein suspended in 0.025 *M* K_2HPO_4, pH 7.2. Dithiothreitol was added to a final concentration of 2.0 m*M* and reactions were initiated by the addition of vitamin K epoxide to a final concentration of 0.010 m*M*. Reactions were performed under argon atmosphere as described previously[6]. Enzymatic reactions (1.0 ml) containing 2 m*M* dithiothreitol were quenched by incubation for 10 min at 40°C in the presence of 5.0 m*M* N-ethylmaleimide[7] and sufficient concentrated potassium hydroxide to bring the pH to 8.0. Volumes were adjusted to 2.5 ml by the addition of 0.025 *M* K_2HPO_4. Vitamins were extracted twice with 3.0 ml of chloroform–methanol (2:1, v/v) by mixing with a vortex mixer at 5.0 min intervals (2×) and centrifuging at 500 *g*.

HPLC determination of vitamins

The chloroform layers containing the extracted vitamins were removed, pooled, evaporated to dryness at 25°C under nitrogen, and dissolved in 0.20 ml of absolute ethanol. Samples (10–50 μl) were injected onto a Waters Model 6000A HPLC equipped with a Nova-Pak C_{18} Radial Pak-L liquid chromatography cartridge. Samples were eluted with a convex gradient (Waters Solvent Programmer Model 660, curve 3) of 10-min duration at a flow-rate of 1.5 ml min^{-1} and detected at 254 nm. The gradient was from ethanol–water (90:10, v/v) to ethanol–hexane (90:10, v/v). After each elution, the column was checked for adhering vitamin by injection of chloroform (100 μl) on the reverse gradient. The column was re-equilibrated at the initial conditions for 10 min prior to reinjection of the next sample. Both vitamin K and vitamin K epoxide gave linear response at 254 nm over the range of 0.02–10.0 nmol. Recovery of both vitamin K and vitamin K epoxide from the column was determined to be essentially complete (greater than 95%) by spectrophotometric assay of the eluted vitamins. All glassware was either disposable or acid washed. All manipulations were performed under subdued light.

RESULTS AND DISCUSSION

Non-enzymatic reaction of vitamin K with dithiothreitol on HPLC columns

Material slightly less polar than vitamin K dihydroquinone eluted on initial

attempts to chromatograph organic extractions of the vitamins in the presence of dithiothreitol (Fig. 1a).

The intensity of this peak was independent of enzyme concentration, time, and temperature of incubation. In addition, this peak could be produced by injection of dithiothreitol (0.001 mmol) together with vitamin K (10–60 nmol) (Fig. 1b) on three separate Radial-Pak C_{18} columns. This peak was not observed on injection of these amounts of vitamin K alone, nor could it be produced on injection of dithiothreitol (0.001 mmol) together with vitamin K epoxide (5–200 nmol). Fig. 1c shows a typical elution profile of the vitamin K compounds for comparison.

To ascertain that the reaction occurred on the HPLC column, a number of experiments were performed in which dithiothreitol was mixed with either vitamin K or vitamin K epoxide, followed by removal of the dithiothreitol and analysis by gradient elution HPLC. Ratios of dithiothreitol to vitamins and experimental conditions (*e.g.*, temperature, pH, time of reaction, etc.) were chosen to approximate those used in the enzymatic assays. Vitamin concentrations ranged from 0.010 to 0.500 m*M*. Under none of these conditions could a reaction between vitamin K and

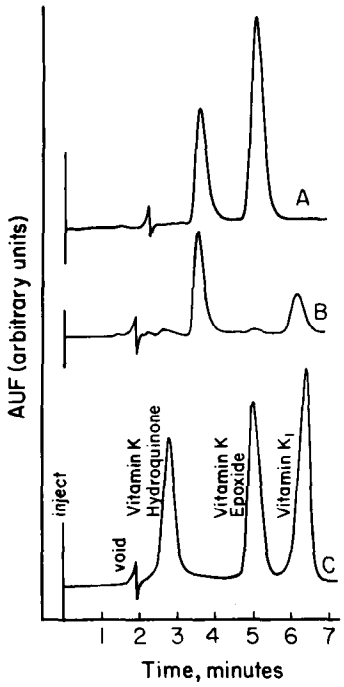

Fig. 1. Formation of non-enzymatic product by reaction of dithiothreitol and vitamin K on HPLC columns. (a) Appearance of non-enzymatic product on isocratic HPLC elution following extractions of vitamins from vitamin K epoxide reductase reaction mixtures containing 2 m*M* dithiothreitol, 0.010 m*M* vitamin K epoxide and 6.0 mg of protein in a final volume of 1.0 ml. (b) Appearance of non-enzymatic products on injection of dithiothreitol (0.001 mmol) followed by vitamin K (10.0 nmol). (c) Isocratic elution profile of authentic vitamin K dihydroquinone, vitamin K epoxide and vitamin K. Enzymatic assays and organic extractions were carried out as described in Methods except that N-ethylmaleimide was not added to remove unreacted dithiothreitol. HPLC assay was on a Waters C_{18} Radial Pak 10 using ethanol–water–H_3PO_4 (97:2.9:0.1, v/v) at a flow-rate of 1.5 ml min^{-1}. Detection was at 254 nm.

dithiothreitol in 0.33 *M* K_2HPO_4 be observed over a pH range 7.2–12.0. Similarly, no reaction could be detected between either vitamin K or vitamin K epoxide and dithiothreitol in excesses of from 5 to 300 fold in 2-propanol at 35°C or in ethanol over a temperature range of 25°C to 80°C. Therefore, we concluded that the reaction we observed occurred on the C_{18} HPLC column.

Spectral analysis of the collected, non-enzymatic products revealed a poorly-defined spectrum with a maximum absorbance at 250 nm. No reduced thiol could be detected using DTNB (5,5'-dithiobis-2-nitrobenzoic acid)[7]. Rechromatography yielded at least four species in various amounts. In view of the instability of this mixture, further chemical characterization was not attempted.

Quantitation of vitamins recovered from the column revealed that loss of vitamin by adsorption on the column occurred over time even though linear standard curves were obtained initially. This loss of the vitamins as well as appearance of the spurious peak worsened with usage. With time (100–200 injections) as much as 50% of the vitamin injected was retained on the column when isocratic elution conditions were used. Therefore, a likely explanation of our results is that reactions between adsorbed vitamin K on the C_{18} column and dithiothreitol are initiated under pressure.

Removal of dithiothreitol with N-ethylmaleimide

Because the reaction between dithiothreitol and vitamin K on the HPLC columns produced a spurious product which, owing to its retention time, interferes with chromatography of the K vitamins (Fig. 1), a procedure using 5 m*M* N-ethylmaleimide prior to vitamin extraction and chromatography was developed. This method effectively removes unreacted dithiothreitol from enzymatic reaction mixtures (Table I). As much as 30% of the dithiothreitol added to reaction mixtures was extracted into chloroform if N-ethylmaleimide treatment was omitted. This procedure has additional advantages in that N-ethylmaleimide effectively quenches the reaction (un-

TABLE I

REMOVAL OF DITHIOTHREITOL FROM EXTRACTED REACTION MIXTURES WITH N-ETHYLMALEIMIDE

Vitamin K epoxide reductase reaction mixtures containing 6.0 mg of protein, 0.010 m*M* vitamin K epoxide and 2 m*M* dithiothreitol in a total volume of 1 ml were quenched, treated with N-ethylmaleimide and extracted into chloroform as described in Methods. To determine the amount of monothiol extracted into the chloroform layer, the layers were evaporated to dryness under nitrogen and the residue was dissolved in 0.25 *M* K_2HPO_4, pH 7.2. Concentrations of monothiol remaining were then determined by treatment with 0.10 *M* 5,5'-dithiobis-2-nitro-benzoic acid as described by Ellman[7] using a molar extinction coefficient of $1.36 \times 10^4\ M^{-1}\ cm^{-1}$ at 412 mm.

N-Ethylmaleimide (mM)	*Monothiol extracted (μmol)**
0.0	0.59
0.5	0.55
1.0	0.31
2.0	0.13
5.0	0.00

* Averages of duplicate assays.

published observations), does not react with the vitamins (Fig. 2), elutes in the void volume and is not corrosive to the HPLC equipment. By comparison, use of millimolar concentrations of mercuric salts to remove dithiothreitol was corrosive to both HPLC pumps and lines.

Extraction of vitamins

Avoidance of the spurious product formed on the HPLC column requires that dithiothreitol is removed prior to assay by HPLC, that the vitamins are effectively extracted into the organic phase, and that the vitamins are quantitatively recovered from the columns. All three vitamins were effectively extracted (greater than 95%)

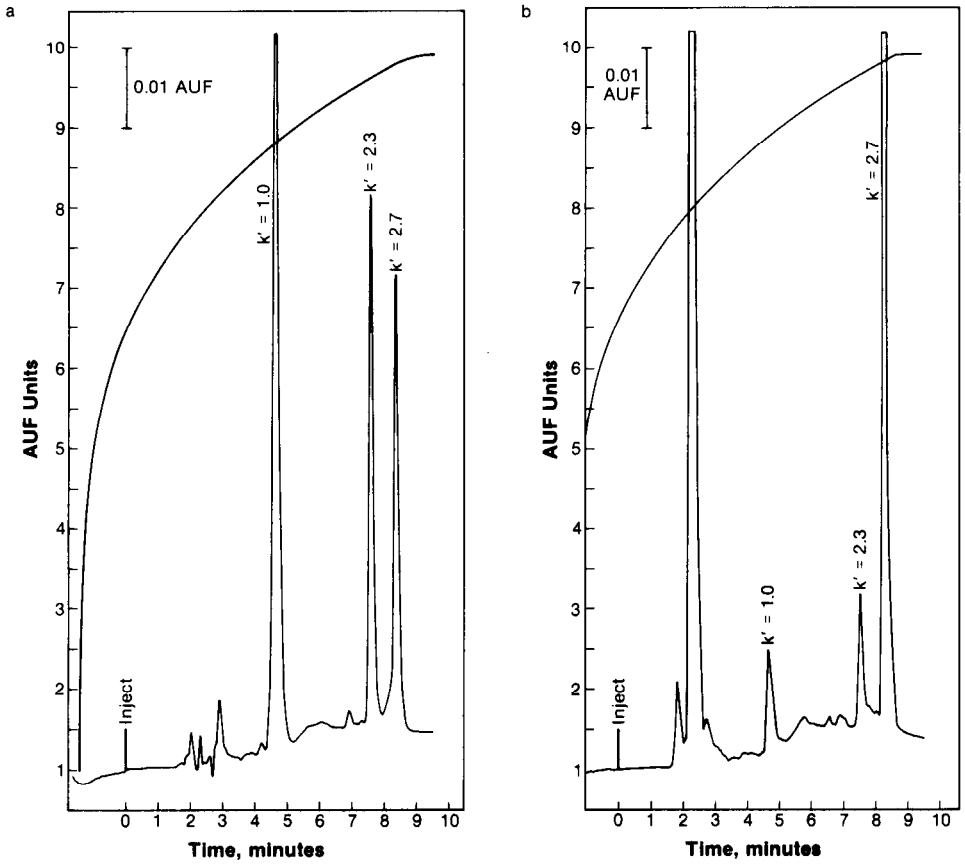

Fig. 2. Gradient elution profiles of: (a) authentic vitamin K dihydroquinone (*ca.* 1.8 nmol), vitamin K epoxide (6.1 nmol) and vitamin K (1.7 nm); (b) vitamin K dihydroquinone (*ca.* 0.10 nm), vitamin K epoxide (1.3 nmol) and vitamin K (5.2 nmol) extracted from vitamin K carboxylase (epoxidase) reaction mixtures containing 10 m*M* dithiothreitol after 15 min. Enzymatic reaction mixtures containing 2.5 mg of protein, 40 μM vitamin K dihydroquionone, 10 m*M* dithiothreitol, 0.5 m*M* peptide substrate and 0.1% Renex 30 in a total volume of 0.5 ml were quenched and treated with N-ethylmaleimide as described in Methods. Extraction of vitamins and gradient HPLC elution were as described in Methods. Species eluting with $k' = 1.0$, 2.3, and 2.7 are vitamin K dihydroquinone, vitamin K epoxide and vitamin K, respectively. Curved line traces the solvent gradient. (Elution trace precedes the gradient trace by 1.5 min).

TABLE II

EXTRACTION OF VITAMIN K SPECIES USING HEXANE AND CHLOROFORM EXTRACTION SYSTEMS*

Vitamins were added to 0.025 *M* K_2HPO_4, pH 7.2 and extracted using either 2-propanol–hexane–0.025 *M* K_2HPO_4 (2:1:1, v/v) or chloroform–methanol–0.025 *M* K_2HPO_4 (2:1:2.5, v/v), as described in Methods. Concentrations of vitamins extracted into 2-propanol–water or methanol–water were determined by difference spectroscopy in the UV using either 2-propanol–water or methanol–water as reference.

	Vitamin K epoxide	*Vitamin K*	*Vitamin K dihydroquinone*
Nanomoles added	105.0	106.0	103.0
Nanomoles remaining**			
Hexane extraction system	5.1	6.8	12.5
Chloroform extraction system	0.0	0.0	0.0

* Averages of duplicate experiments. Numbers refer to vitamin species remaining in the alcohol-water phases and hence not extracted.

** Lower limit of sensitivity, 0.001 nmol.

from phosphate buffers with chloroform extraction systems (Table II). The incomplete extraction obtained with hexane systems, a commonly used procedure[6], is shown for comparison. The efficiency of extraction of the vitamins with chloroform did not vary with the concentration of phosphate buffer over the range 0.025–0.75 *M*.

Assay of vitamins by gradient elution HPLC

A gradient procedure was developed for the assay of the extracted vitamins (Fig. 2). With this system, vitamin K, vitamin K epoxide and vitamin K dihydroquinone were resolved. Both vitamin K epoxide and vitamin K were effectively eluted (greater than 95%) as measured by spectrophotometric assay of the collected vitamins. None of the vitamin K species was completely eluted from radial compression C_{18} columns using a variety of solvent systems when final solvent polarities were greater than $\rho = 5.0$[8]. In our low polarity gradient system, the use of 0.1% H_3PO_4, a commonly used ion-pairing agent[9], caused rapid deterioration of radial compression C_{18} columns.

With the gradient elution procedures described in Fig. 2, picomolar amounts of the K vitamins can be detected. Thus both vitamin K-dependent carboxylase (epoxidase) and vitamin K epoxide reductase can be quantitatively assayed. No non-enzymatic reaction of either vitamin K or vitamin K epoxide with dithiothreitol could be detected at sensitivities of 0.02 a.u.f.s. Vitamin K dihydroquinone is readily extracted by this method (Fig. 2a and b). However, the method will not allow quantitation of the dihydroquinone owing to variability in extent of its oxidation during the extraction procedure. Recovery of the vitamins from enzymatic reaction mixtures varies inversely with the protein concentration. This is important for the assay of vitamin K epoxide reductase, where crude microsomes are commonly used as the source of the enzyme[1]. Low recovery of the vitamins precludes reliable assay of reaction mixtures containing greater than 10 mg ml^{-1} protein.

Preusch *et al.*[3] have reported a non-enzymatic reaction of vitamin K epoxide to form vitamin K and vitamin K dihydroquinone. Indeed, free radical reactions and reactions of thiolate anions with quinones could be envisioned[2], particularly in complex mixtures of crude microsomes and impure detergents. Therefore, care must be taken to avoid side reactions of the vitamins with dithiothreitol when interpreting data in terms of enzymatic pathways and mechanisms.

In general, it has been assumed that HPLC columns are inert and that the method is non-destructive to the K vitamins[4]. However, the results presented here show that vitamin K can react with dithiothreitol on C_{18} HPLC columns. In fact, this probably should be expected, considering the potential for surface catalysis. Therefore, this possibility must be considered in quantitatively interpreting HPLC analysis of vitamin K species. The observation that vitamin K species can be "trapped" on HPLC columns should be noted and could have some practical value. Lochmüller and Wilder[10] for example, have utilized the observation that *n*-alkanes can become stably entrained on C_{18} columns to characterize bonded phases. As it is likely that under our conditions, vitamin K is similarly trapped on the C_{18} columns by virtue of its phytyl chain, the quinone moiety could be favorably positioned for reaction on the column. Adsorption of the vitamins to hydrophobic matrices of this type could, in addition, have some utility for the study of the vitamin K-dependent enzymes. A theoretical argument based on such a model has been used to explain the function of the phytyl chain as an "anchor" for the proper positioning of the quinone moiety in biological membranes[11].

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