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CHEMICAL REDUCTION SYSTEM FOR THE DETECTION OF PHYLLOQUINONE (VITAMIN K_1) AND MENAQUINONES (VITAMIN K_2)

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SUMMARY

Both isocratic and gradient elution systems for fluorometric detection of K vitamins after post-column reduction with zinc metal to their hydroquinones are described. The reaction detection system for K vitamins (phylloquinone and menaquinones) in liquid chromatography is based on reduction of K vitamins to their corresponding hydroquinones with zinc metal in the presence of zinc ions. It was found that 95% of the injected quinones (K vitamins) could be reduced to their corresponding hydroquinones with zinc metal compared to 60% reduction for electrochemical detectors. Menaquinones could be detected down to 100 pg with relative ease during gradient elution.

INTRODUCTION

High-performance liquid chromatography (HPLC) has become a standard method for the analysis of vitamin K in a wide variety of biological samples[1–9]. A major drawback of previously developed methods has often been the inadequacy of the systems employed to detect selectively and sensitively the low physiological concentration of K vitamins. As a consequence, most assays for vitamin K require no less than two chromatographic steps to increase the selectivity of separating vitamin K from contaminants present in most biological lipid extracts. To increase the sensitivity for the detection of K vitamins in biological samples it is, moreover, necessary to process large sample volumes and gram quantities of food products[1,2,8,9].

Although these problems have to a certain extent been overcome by the recent introduction of an electrofluorometric assay for phylloquinone (K_1) which involves post-column electrochemical reduction of K_1 to vitamin K_1 hydroquinone[6], these methods suffer from incomplete reduction of the injected K vitamins[10]. In addition, the complete removal of oxygen is essential for both efficient electrochemical reduction and elimination of fluorescence quenching[6,10,11].

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The purpose of this paper is to report on the development of a selective and sensitive chemical method which reduces 95% of the injected K_1 to its corresponding hydroquinone and thus eliminates the requirement for electrochemical reduction of K_1 prior to fluorescence detection. The method described also removes oxygen from the mobile phase to enhance fluorometric detection of K_1 hydroquinone.

EXPERIMENTAL

Chemicals

Synthetic phylloquinone (2-methyl-3-phytyl-1,4-napthoquinone; K_1) was obtained from a commercial source (Sigma, St. Louis, MO, U.S.A.). Menaquinones (MK 4–10) were gifts from M. J. Shearer (Guy's Hospital, London, U.K.). Phylloquinone 2,3-epoxide (K_1 epoxide) was synthesized as described previously[12]. HPLC-grade solvents were obtained from Burdick & Jackson (Muskegon, MI, U.S.A.).

HPLC

The liquid chromatograph consisted of either a Model 510 reciprocating pump (Waters Assoc., Milford, MA, U.S.A.) or a Perkin-Elmer (Norwalk, CT, U.S.A.) Model Series 3B pump which was connected to an autosampler (WISP, Waters) fitted with a C_{18} pre-column (30 × 4.6 mm I.D.; Rainin Instruments, Woburn, MA, U.S.A.). For pre-column reduction the electrochemical cell (Model 5100; Environmental Science Assoc., Bedford, MA, U.S.A.) or the zinc reducer column was inserted between the pre-column and the analytical column (C_8, Microsorb; 100 × 4.6 mm I.D.; Rainin). The analytical column was connected to a UV detector (Model LL-85B; Perkin-Elmer). For post-column reduction, the electrochemical cell or the zinc reducer column was placed between the analytical column (Hypersil ODS; 250 × 4.6 mm I.D.; Shandon Southern Products, Sewickly, PA, U.S.A.) and a fluorometer (Model 970 or Model 980; Kratos Analytical Instruments, Ramsey, NJ, U.S.A.).

Solid-phase reactor

High-purity 200-mesh zinc particles (Alfa Products, Danvers, MA, U.S.A.) were dry-packed into 20 × 3.9 mm I.D. stainless-steel columns, using 0.5-μm stainless-steel frits.

RESULTS AND DISCUSSION

General principles

The principle of the vitamin K reducer column is based on the earlier finding that 95% of the injected K_1 could be reduced to the corresponding hydroquinone (K_1H_2) when in contact with zinc metal in the presence of zinc ions[10].

$$Zn \rightarrow Zn^{2+} + 2e^- \quad (1)$$

$$2H^+ + 2e^- + K_1 \rightarrow K_1H_2 \quad (2)$$

$$Zn + 2H^+ + K_1 \rightarrow Zn^{2+} + K_1H_2 \quad (3)$$

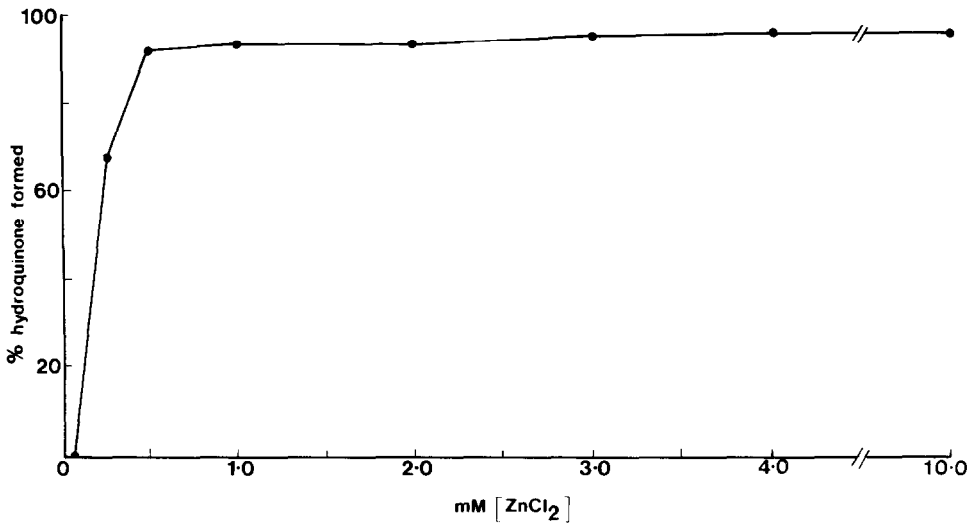

Fig. 1. Reduction efficiency for K_1 *vs.* zinc ion concentration. Column: Microsorb C_8; mobile phase, 95% methanol containing aqueous zinc chloride (pH 3.3); detection, 254 nm; flow-rate, 1.0 ml/min.

This finding was initially made while developing an electrofluorometric assay for K_1, which involved post-column electrochemical reduction of the quinone, followed by fluorometric detection of the generated hydroquinone. It was subsequently found that a similar reaction occurred when K_1 was allowed to react with zinc metal in the presence of zinc ions[10].

Selection of reaction conditions

In order to optimize the reaction conditions with respect to zinc ion concentration, a zinc reducer column was inserted between the analytical column and the injector, and the concentration of zinc chloride in a mobile phase of 95% aqueous methanol (pH 3.3) was varied. After separating the injected K_1 from the generated K_1H_2, it was found that as the concentration of zinc chloride was raised from 0.06 m*M* to 0.25 m*M* while maintaining the pH at 3.3, the reduction efficiency could be increased from 0 to 67% (Fig. 1). Maximum reduction (95%) was achieved between 1 and 10 m*M* zinc chloride. The maximum reduction efficiency remained constant as the pH of the mobile phase was varied between 2.1 and 4.5.

Flow-rate

The effect of decreasing the residence time of K_1 in the zinc reducer column as a function of K_1H_2 formation was examined by increasing the flow-rate from 0.5 to 2.0 ml/min. It was found that the reduction efficiency remained constant (95% K_1H_2 formed) with increasing flow-rate in this range.

Reductive efficiency

The efficiency of the zinc reducer column in reducing K_1 over a range of K_1 concentration was evaluated by injecting 5 ng to 2 μg K_1 "on-column". After separating K_1 from K_1H_2, it was found that within this range of concentrations, 95% of the injected quinone was consistently reduced to K_1H_2.

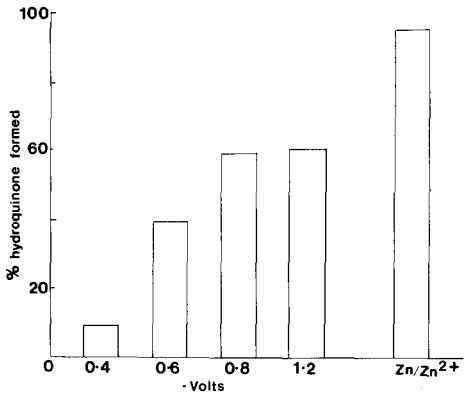

Fig. 2. Comparison of electrochemical and chemical reduction efficiencies for K_1. For conditions, see Fig. 1.

Comparison with electrochemical reduction

In order to compare the efficiencies of K_1 reduction between the zinc reducer column and previously developed electrochemical reduction methods[6,7], the zinc reducer column was replaced by a dual-electrode porous-graphite electrochemical cell. After separating K_1 from K_1H_2, it was found that at the peak plateau voltage (-0.8 V) for K_1, about 60% of the injected K_1 was reduced to K_1H_2 (Fig. 2). In analogous experiments with the zinc reducer column, 95% of K_1 was converted to K_1H_2 (Fig. 2). A possible reason for the incomplete electrochemical reduction of K_1 may be due to a coupled electrochemical reduction reaction preceding the reduction of K_1.

Electrode modification

In experiments in which the zinc column was inserted between the pump and the injector to scavenge oxygen[11] while K_1 was reduced electrochemically, it was found that the amount of hydroquinone generated (95%) remained constant as the

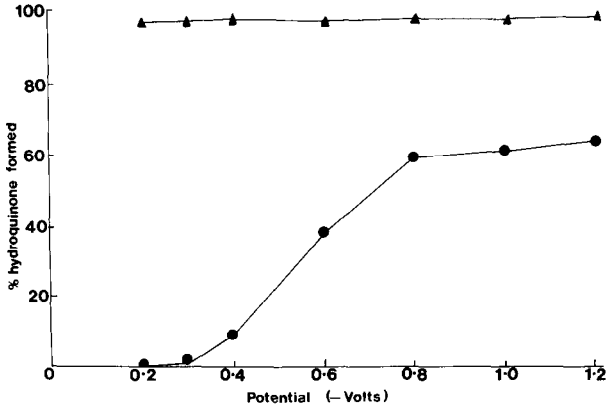

Fig. 3. Hydrodynamic voltammograms for K_1 after pre-column electrochemical reduction in the presence (▲—▲) and absence (●—●) of zinc metal. For conditions, see Fig. 1.

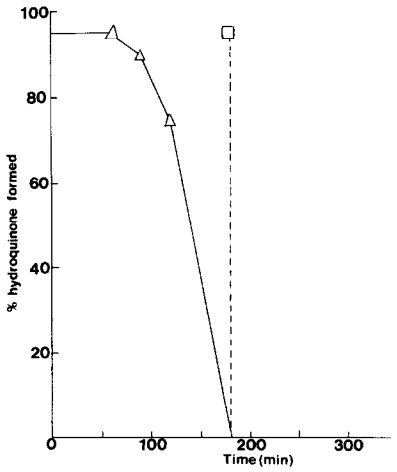

Fig. 4. Time-dependent loss of K_1 reduction at 0.00 V (△—△) and regeneration of reduction efficiency at −0.2 V (dashed line). For conditions, see Fig. 1.

reduction potential was changed from −0.2 to −1.2 V. These results were in contrast to the finding that in the absence of zinc, a peak plateau for K_1H_2 was reached at −0.8 V (Fig. 3). In later experiments, an observation was made which suggested that reduction was possible even if no potential is applied, though the reduction efficiency decreased over a period of several hours (Fig. 4). After the reduction efficiency had decreased to 0% K_1H_2 formation, it could be regenerated by applying a potential of −0.2 V (Fig. 4).

At potentials greater than −0.8 V zinc ions in the mobile phase may be reduced to zinc metal on the surface of the electrode, which then reacts with K_1, reducing it

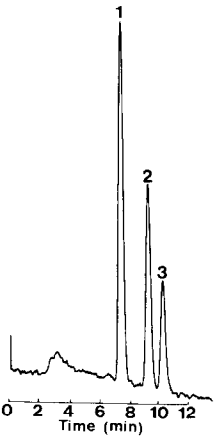

Fig. 5. Separation of vitamin K compounds by reversed-phase HPLC on Hypersil ODS. Mobile phase: 20% dichloromethane in methanol containing 10 m*M* zinc chloride and 0.1 *M* acetic acid–sodium acetate (pH 4.5); detection, 248 nm ex., 420 nm em.; flow-rate, 1.0 ml/min; peaks: 1 = K_1 epoxide (0.9 ng); 2 = K_1 (0.5 ng); 3 = $K_1(I\text{-}H_2)$ (= K_1 with the 2′,3′ double bond hydrogenated) (0.25 ng).

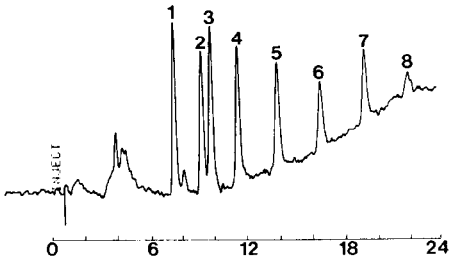

Fig. 6. Separation of vitamin K compounds (200 pg each) by reversed-phase HPLC on Hypersil ODS by gradient elution. Mobile phase A, 20% dichloromethane in methanol containing 10 m*M* zinc chloride; mobile phase B, 40% dichloromethane in methanol containing 10 m*M* zinc chloride and 0.1 *M* acetic acid–sodium acetate (pH 4.5); linear gradient 0 to 100% B in 20 min; detection, 248 nm ex., 420 nm em.; flow-rate, 1.0 ml/min; peaks: 1 = MK-4; 2 = MK-5; 3 = K_1; 4–8 = MK-6–MK-10.

and being oxidized to zinc ions (reaction 3) However, the precise nature of events leading to the reduction of K_1 at -0.2 V is not clear, as at this potential no reduction of zinc ions would take place. Reduction of K_1 at -0.2 V could be eliminated by applying a potential of $+0.9$ V.

Application of the zinc reducer column for post-column reduction of vitamin K compounds

The fluorometric detection of K_1 and related compounds after post-column reduction with zinc to their corresponding hydroquinones is shown in Fig. 5. Chromatography was performed on a reversed-phase column (Hypersil ODS) with a mobile phase consisting of 20% dichloromethane in methanol and containing 10 m*M* zinc chloride.

The major advantage of this mode of detection lies in the increased sensitivity that can be obtained for the detection of K_1 epoxide and K_1. The lower limits of detection for these compounds were found to be 25 pg compared to 500 pg for a UV

TABLE I

CAPACITY RATIOS (k') OF K VITAMINS BY REVERSED-PHASE HPLC AND ISOCRATIC ELUTION ON HYPERSIL ODS

Mobile phase 20% dichloromethane in methanol, containing 10 m*M* zinc chloride.

Compound	k'
MK-4	0.9
K_1 epoxide	1.2
MK-5	1.5
K_1	1.7
$K_1(I\text{-}H_2)$	2.0
MK-6	2.2
MK-7	3.3
MK-8	4.8
MK-9	7.1
MK-10	10.4

photometer, 100 pg for electrochemical detection, and 150 pg for chemical reduction[13,14]. In addition, the chromatographic system reported here overcame the solvent restrictions imposed during electrochemical reduction, which requires an eluent that can dissolve the supporting electrolyte. As a consequence, such methods rule out the use of the highly efficient reversed-phase systems, which require non-aqueous mobile phases for the separation of K vitamins[15,16].

The capacity ratios for MKs 4–10 and K_1 are shown in Table I. This separation was achieved with an isocratic mobile phase of 20% dichloromethane in methanol containing 10 m*M* zinc chloride (pH 4.7). A linear relationship between log of capacity factors for MKs 4–10 and the carbon number of the side-chain was observed.

A chromatogram obtained during gradient elution of 200 pg each of MKs 4–10 is shown in Fig. 6. The figure illustrates the use of high sensitivity gradient methods for the detection of sub-nanogram levels of MK. It was found that an increase in the dichloromethane content of the mobile phase to 40% during gradient elution caused considerable quenching of fluorescence and that 200 pg of MK-10 was barely detectable (Fig. 6). However, the systems described here constitutes a considerable enhancement in selectivity, sensitivity, and stability over electrochemical and chemical reduction methods for the detection of MKs having up to nine isoprenoid units.

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