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Determination of vitamin K_1 in serum using catalytic-reduction liquid chromatography with fluorescence detection

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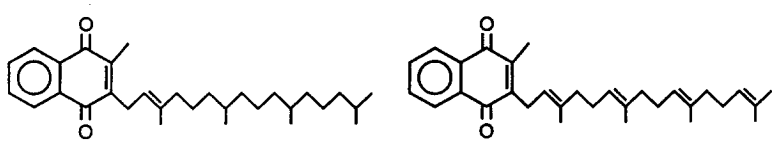
Abstract

A new method for the liquid chromatographic–fluorescence determination of serum vitamin K_1 is described using reduction of the K-quinone to the fluorescent K-hydroquinone. The reduction reaction occurs "on-line" in the LC system using a catalytic reducer column and an alcohol mobile phase as reductant. A procedure for serum determination utilizes a liquid–liquid serum lipid extraction followed by normal-phase fractionation on a solid-phase extraction cartridge. The final measurement uses a reversed-phase (C_{18}) separation with a ethanol–methanol mobile phase and provides a detection limit of approximately 20 pg/ml.

1. Introduction

The vitamin(s) K are substituted 4-methyl-naphthoquinones with a *trans* double bond isoprene link to additional isoprene-derived side chain units:

The importance of the determination of vitamin K_1 stems from its dietary requirement as a co-factor for the formation of prothrombin and other blood-clotting proteins by the liver, its role in calcium-binding protein genesis and for its possible effect on the respiration of malignant



Vitamin K_1 (phylloquinone)

Vitamin K_2 (menaquinone-4)

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cells [1]. Although nutritional deficiencies are uncommon in healthy individuals, serum vitamin K_1 may be reduced in cases of extreme malnutrition and in premature babies [2].

In order to investigate the biological function, metabolism and the effect of diet on serum vitamin K_1 levels, sensitive and accurate assays are needed. There has been a poor consensus on the "normal" physiological concentration of serum vitamin K_1, with reported mean values as low as 100 pg/ml and as high as 4 ng/ml [3]. Although some of this disagreement may be the result of dietary influences on serum vitamin K_1 levels, much of this disagreement is a result of lack of sensitivity and selectivity of the analytical methods and the lack of available standard reference sera for vitamin K_1 method validation. Early LC methods, based on UV–Vis absorbance detection, required extensive sample preparation, clean-up and concentration before measurement [1,4] and do not provide the sensitivity required to assay serum from vitamin K_1-deficient individuals. Recently, more sensitive methods, based on the reduction of the K-quinone moiety to the hydroquinone, have become prominent. Electrochemical detection has been investigated using direct reductive amperometry [2], reductive coulometry and coulometric dual-electrode reoxidation of the electroreduced K-hydroquinone [5] with detection limits determined to be 300, 250 and 150 pg, respectively. However, addition of mobile-phase electrolytes and rigorous exclusion of oxygen from the mobile phase and samples was required for optimal performance with these electrochemical approaches. LC, using fluorimetric detection of the K-hydroquinone following reduction, also provides excellent sensitivity for vitamin K_1 determination, with reported detection limits as low as 20 pg [6]. Off-line chemical reduction with sodium borohydride has been used for vitamin K_1 determinations in feces [7] and serum [3]. A number of LC-fluorescence methods for serum vitamin K_1 determination have used on-line reduction using reductive coulometry [5,6,8–10], post-column, wet-chemical reduction with borohydride [3], solid-chemical reduction with a metallic zinc reducer column [11], catalytic chemical reduction with hydrogen [12] as well as catalytic [13] and photo-reduction [14,15] using alcohol mobile phases. For optimal performance, all of these on-line methods require removal of mobile-phase oxygen, either by sparging with inert gas or using on-line chemical reduction following the LC pump. Coulometric reduction requires addition of corrosive electrolytes and the efficiency may decline with time via electrode fouling [5]. Photo-reduction requires mobile-phase and sample oxygen sparging. In addition, the photo-reactor coil adds some peak dispersion. Our experience with the zinc reducer system for the detection of nitro-polycyclic aromatic hydrocarbons (NPAH) [16–18] showed that a mobile-phase electrolyte must be used to support this "electroless" zinc reduction. In addition, a more serious difficulty occurs upon exhaustion of the supply of zinc in the reducer column, as it is consumed by oxygen and the analyte reduction. The shrinking zinc particles pass through the reducer column end frits, limiting the useful life of the reducer and releasing damaging particles into the LC system. This drawback inspired a new approach to on-line mobile-phase oxygen-reduction using an alcohol in the presence of a platinum-on-alumina catalyst [19].

Two previous methods for vitamin K_1 determination have been based on catalytic reduction either by the use by hydrogen-saturated mobile phases at a reduced platinum oxide catalyst [12] or by catalytic alcohol reduction at powdered platinum-black [13]. Both methods require mobile phase sparging, either for hydrogen saturation or oxygen removal, respectively, adding complexity and operational difficulty to the LC system.

In this paper, we investigate the use of a platinum-on-alumina catalyst/alcohol reduction approach to the LC fluorescence determination of vitamin K_1 in human serum. The on-line reduction does not require either sparging or addition of a mobile-phase electrolyte, provides excellent reduction at room temperature, and uses a commercially available, ready-to-use catalyst. The new method uses liquid–liquid serum extraction followed by normal-phase fractiona-

tion on a solid-phase extraction cartridge for removal of excess lipids and to reduce the matrix fluorescence background.

2. Experimental[2]

2.1. Chemicals

Vitamins K_1 (2-methyl-3-phytyl-1,4-naphthoquinone) and K_2 (Menaquinone-4) were obtained from Sigma (St. Louis, MO, USA). Three catalyst materials were tested for the reduction of the K vitamins: 10% platinum-on-carbon, 10% platinum-on-alumina and platinum(IV) oxide (all obtained from Alfa Products, Danvers, MA, USA). The catalysts were dry-packed, aided by the use of a 60-Hz vibrator, into 50×4 mm column blanks with 2-μm stainless steel end frits. *Caution should be exercised in the handling and disposal of these active catalysts since contact of flammable solvents with small quantities of catalyst can result in immediate combustion.*

2.2. Extraction of vitamin K_1 from serum

Serum was prepared for vitamin K_1 determination by a liquid–liquid lipid extraction followed by fractionation on a solid-phase extraction (SPE) cartridge. Frozen serum was warmed to room temperature, vortexed for 5 s, and 500 μl was measured into a conical centrifuge tube. An aliquot of 1 ml of an approximately 1 ng/ml solution of internal standard, vitamin K_2 in 2-propanol, was added and vortexed for 5 s to precipitate serum proteins. The sample was then extracted three times with 2 ml of hexane by vortexing for 30 s. Centrifugation (5 min at 2000 *g*) was used to separate the layers following each extraction. The supernate was subsequently withdrawn, combined in an amber vial, and dried under a stream of nitrogen, taking care to remove all of the co-extracted alcohol. The sample was redissolved for SPE fractionation by thoroughly vortexing with 500 μl of hexane. Silica SPE cartridges (Enviroprep Inert SPE, 500 mg, Baxter Healthcare Products, Muskegon, MI, USA) were used for the isolation of the K vitamins from matrix interferences. New cartridges are washed with 5 ml of hexane prior to sampling. The extract is then sorbed to the column, and eluted by gravity using 10 ml of hexane, which is discarded. The K vitamin fraction is eluted with 5 ml of 3% ether–hexane. The first 1 ml that elutes is discarded and then the subsequent 4 ml is collected for further processing. Cartridges may be reused by washing with 10 ml of 3% ether–hexane followed by 10 ml of pure hexane.

The 4 ml eluate was then dried under nitrogen, and the residue redissolved in a volume of ethanol that is determined by the sensitivity of the fluorimetric detector. Reconstitution volumes of 100 and 500 μl were used for high sensitivity and routine analysis, respectively. Care must be taken to swirl the alcohol around the sides of the vial to insure complete recovery of the vitamins. A sample volume of 50 μl was then injected into the chromatograph for analysis.

2.3. Analytical reversed-phase separation and detection

For the analytical separation, a reversed-phase, C_{18} bonded-phase silica column (201TP 54, 25×0.46 cm, 5-μm particles, Vydac, Hesperia, CA, USA) was used with isocratic ethanol–methanol solvent mixtures. HPLC-grade solvents were used for all studies. The final method used ethanol–methanol (40:60, v/v) mobile phase with a flow-rate of 1 ml/min.

In order to evaluate the efficiency of the catalytic reducer columns, they were placed in the LC system behind the analytical column but in front of the detector as shown in Fig. 1. For the final vitamin K method, the 10% platinum-on-alumina catalyst was used with a 50×2 mm

[2] Certain commercial equipment, instruments, or materials are identified in this report to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

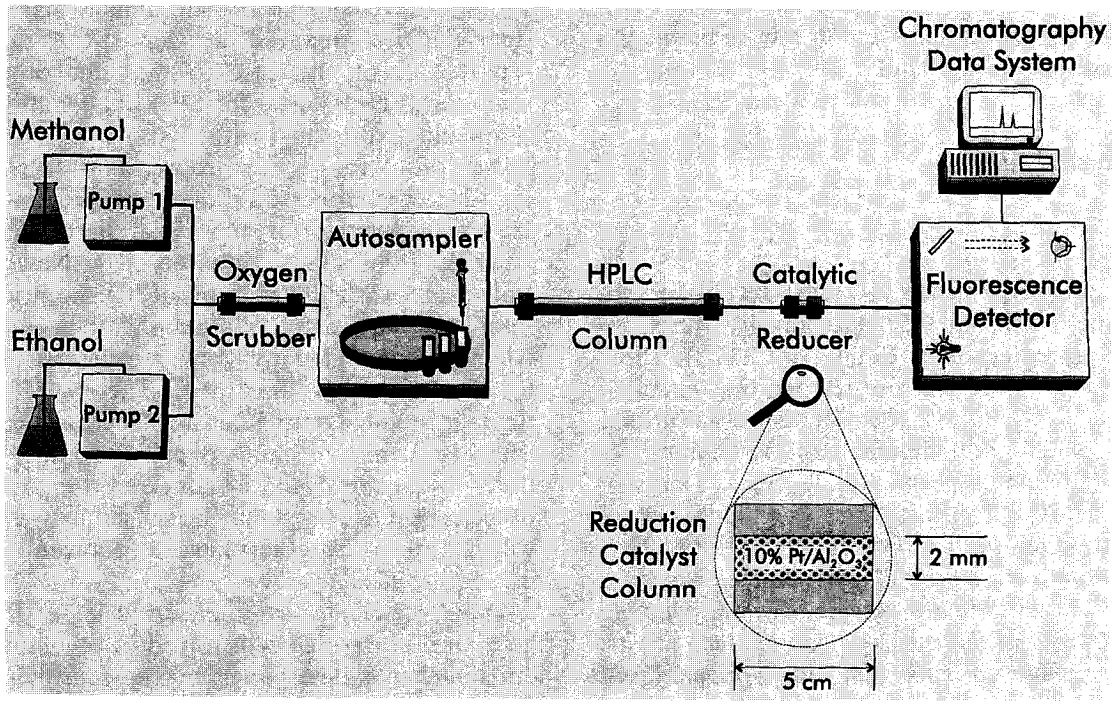


Fig. 1. Block diagram of vitamin K catalytic reduction LC–fluorescence system.

column bed. Optimal vitamin K reduction yield was obtained using mobile-phase oxygen-reduction [19]. The oxygen reducer column (10 × 0.46 cm) was packed with the 10% platinum-on-alumina catalyst and placed in the LC in front of the injector as shown in Fig. 1. Both catalyst columns retained full activity for the 18 months of the study.

The liquid chromatograph with autosampler was of conventional design. A variable, fixed-wavelength UV–Vis absorbance detector was used for the retention and reaction studies. Two LC fluorescence detectors were tested for the detection of the K-hydroquinone reduction product, a deuterium light source, filter fluorometer with variable excitation wavelength (Model FS 970A, Schoeffel Instruments, Rahway, NJ, USA) and a double monochromator instrument with a xenon light source (Model LS 40, Perkin Elmer, Norwalk, CT, USA). The detector wavelengths were 242 nm for excitation and either a cut-off filter (>280 nm) or monochromator (at 430 nm) for emission. An amperometric electrochemical detector (Model 400, EG&G Princeton Applied Research, Trenton, NJ, USA) with a 3-mm diameter glassy carbon electrode was also evaluated for detection limit at an applied potential of +0.4 V (versus a Ag/AgCl, 3 mol/l KCl reference) which provided current on the diffusion plateau of the K-hydroquinone.

2.4. Standardization

It is crucial to add an internal standard to the serum prior to extraction, to correct for volume changes and other losses in the procedure. For this method development work, menaquinone-4 (vitamin K_2) was chosen as internal standard, since it is structurally identical to vitamin K_1 except for unsaturation of the three extant isoprene units. Menaquinone-4 is generally found at much lower levels in human serum than vitamin K_1, whereas the higher menaquinones-*n* (with *n* corresponding to between 5 and 10 additional isoprene units) have been found in appreciable amounts [8,12]. No detectable menaquinone-4

was found in the two serum pools used in this method development exercise. The chromatographic behavior of vitamin K_2 is very similar to vitamin K_1 for both the fractionation and reversed-phase separation steps and was thus suitable as an internal standard. However, for the routine determination of unknown sera that may contain vitamin K_2, a vitamin K_1 analog with a fully saturated side chain has been reported [20]. It is prepared by exhaustive hydrogenation and subsequent reoxidation to the K-quinone. This synthetic analog appears to be the best internal standard for vitamin K LC determinations.

Calibration solutions were prepared by dissolving vitamin K_1 and K_2 in ethanol and were value-assigned by spectrophotometry. The molar absorptivity of vitamin K_1 that was used was 41 000 at 242 nm. Stock solutions of approximately 40 μg/ml were stored in the dark in the refrigerator and diluted just prior to use. The stock solutions appeared to be stable for at least 2 months.

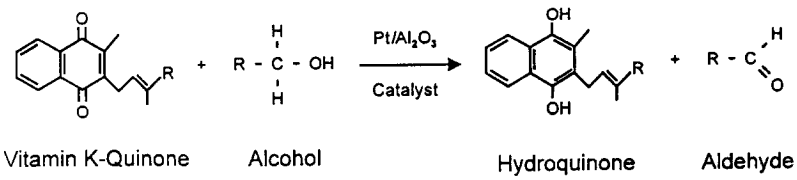
3. Results and discussion

3.1. Catalytic reduction of vitamin K

Several different catalysts were evaluated for the quantitative reduction of the K-quinone functionality to the hydroquinone using alcohols as reductants. Previous work on catalytic mobile-phase oxygen-reduction had shown that 5% platinum-on-alumina provided superior catalysis of the methanol oxidation when compared to either 5% rhodium or 5% palladium-on-alumina [19]. For this work, we tested 10% platinum-on-carbon, platinum(IV) oxide and 10% platinum-on-alumina for the ethanol reduction of the vitamin K_1 and K_2 quinones. Performance of the catalyst column was evaluated by examining the magnitude of the fluorescence signal as an indication of reaction yield. In addition, the effect of the column on peak shape was noted. The platinum-on-carbon completely adsorbed the reactants and products yielding no fluorescence or absorbance signal. Very little reduction of the K-quinones was found for the platinum(IV) oxide catalyst when ethanol was used as reductant at 25°C. We did not test this catalyst with a hydrogen-saturated mobile phase [12] because of the explosion hazard. On the other hand, the 10% platinum-on-alumina gave excellent catalytic reduction efficiency with both methanol and ethanol at room temperature and added only a moderate dispersive effect to the peak shape. This was the catalyst we chose for further study for vitamin K determination.

We investigated the characteristics of the catalytic reduction process in several ways. The reduction products were examined by reversing the reducer and analytical columns in the LC system. In this way the reaction products could be separated and monitored by the absorbance detector at 242 nm. Only a single reduction product was noted. In addition, a few percent of unreacted K-quinone was evident. The reduction product was also collected for off-line spectrophotometry using the catalyst column behind the analytical column using the elution conditions of the method. Care was taken to exclude oxygen to prevent reoxidation of the product back to the K-quinone. Full spectral scans of 200 to 400 nm reveal that the characteristic absorption band of the K-quinone at 242 nm was substantially reduced by the reaction, yet the intensity could be completely restored by allowing the solution to spontaneously reoxidize in air. We could find no spectrophotometric evidence of the reduction of the unsaturated side chain of either vitamin K_1 or K_2, which would have changed the reoxidized spectral from that of the starting materials. A further test of the possibility of reduction of the side chain alkene moiety was done using vitamin K_2 in the LC system. Reduction of any of the four side chain double bonds would produce a product with a retention time more similar to the monounsaturated vitamin K_1. For the test, we collected the vitamin K_2 reduction product as it eluted from the LC, allowed air oxidation of the K-hydroquinone to the quinone, and then reinjected the fraction into an LC system with a UV detector (and no reducer column). The elution time was identical to unreacted Vitamin K_2 and was different from the more saturated vitamin

K_1. Air reoxidation of the product of the on-line vitamin K_1 reduction also resulted in an LC peak that exactly matched the retention of unreacted vitamin K_1. Thus we are convinced that only reduction of the K-quinone moiety to the hydroquinone occurs with the alcohol-based catalytic system and that, unlike the hydrogen catalytic reduction system [12], none of the side chain alkenes are reduced. Based on these experiments and our previous study [19] of this catalytic system, we conclude that the following reaction characterizes the reduction of the K vitamins:



nol–methanol mixtures for the vitamin K separations.

The reversed-phase retention characteristics of the vitamin K_1 and K_2 were determined as a function of the ethanol–methanol composition of the mobile phase, as shown in Fig. 3. The capacity factor was reduced as the proportion of ethanol was increased. For the analytical determination, the mobile phase composition was adjusted to optimize separation of the K-vitamins from early eluting, interfering serum components in a fractionated extract. A final composition of ethanol–methanol (40:60, v/v) was chosen for the serum determinations.

3.2. LC determination

The flow-rate dependance of the fluorescence signal of the hydroquinone product was determined for an ethanol–methanol (20:80, v/v) mobile-phase. A plot of signal versus the inverse of the flow-rate is shown in Fig. 2. High flow rates provide only a limited residence time in the reducer column and gave lower conversion efficiency. A slow flow-rate of 0.35 ml/min appears to provide 100% conversion, but produces very long elution times. Although the reduction yield was only approximately 60% at 1 ml/min, it was found to be quite reproducible and provided reasonable elution times. The catalytic reduction yield was found to be equally high for both methanol- and ethanol-based solvent systems. We also tested mobile phases containing acetonitrile and tetrahydrofuran for the catalytic reduction. Neither solvent provided reduction of the K-quinone. The catalyst was poisoned when concentrations of acetonitrile or tetrahydrafuran above 10% were added to the mobile phase. However, full catalytic activity could be restored by pumping the catalyst column with pure ethanol for one hour. All further studies used ethanol

Fluorescence detection was tested with both deuterium and xenon light source fluorimeters. The linearity of response and the detection limit were determined using a series of standards

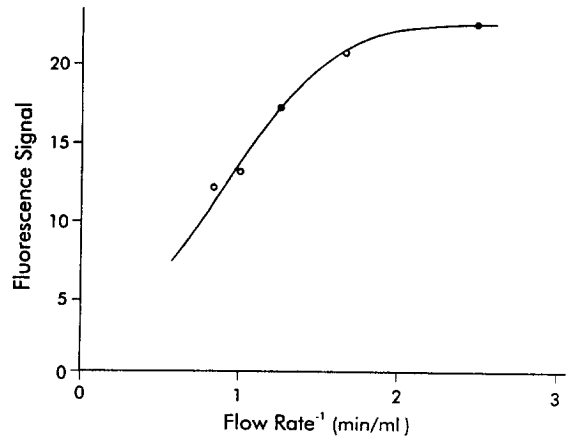


Fig. 2. Flow-rate dependance of fluorescence signal for vitamin K_1. Conditions: mobile phase, ethanol–methanol (20:80, v/v); catalyst column; 5 × 0.2 cm, 10% platinum-on-alumina.

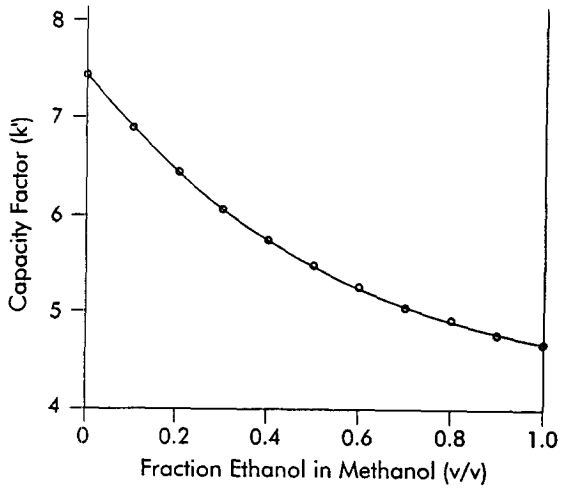


Fig. 3. Reversed-phase retention of vitamin K_1 as a function of ethanol–methanol ratio. Conditions: column, Vydac 201 TP 54 (25 × 0.46 cm, 5 μm); flow-rate, 1.0 ml/min.

providing from 7 to 5000 pg injected. The calibration curves were linear for both detectors and the detection limits (defined by 3 times the peak-to-peak noise) for a 50-μl injection were 50 and 7 pg for the deuterium- and xenon-source fluorimeters, respectively. The lower detection limit is somewhat better than the 25–150 pg recently reported for other reductive/fluorescence detection approaches for vitamin K_1 [5,10,12,15].

We also evaluated oxidative amperometric detection of the hydroquinone at a glassy carbon electrode following the on-line catalytic reduction. The detection limit was found to be approximately 200 pg injected, which was less sensitive than the fluorescence detection, as found previously [5]. All further studies were done using fluorescence detection.

3.3. Vitamin K_1 extraction, fractionation and determination

The serum liquid–liquid extract is much too complex for direct LC determination of vitamin K_1. Co-extracted fats provide a practical limit to the sample concentration that may be achieved during extract solvent exchange. In addition, co-extracted fluorescent serum components, particularly retinol and tocopherols interfere with the detection since they are typically present in 1000- and 10 000-fold excess to vitamin K_1, respectively. Removal of the tocopherols from the crude extract proved to be particularly important because of their similarity in reversed-phase retention as well as fluorescence excitation wavelength to vitamin K_1. As in other published vitamin K methods, a normal phase "open-bed" column is required to selectively fractionate the vitamins before analysis. Several SPE cartridges were tested for the K fractionation. Commercial plastic-envelope cartridges were found to exhibit a high background fluorescence signal, presumably from plasticizer additives. Solvent precleaning did not reduce the fluorescent background sufficiently for the high sensitivity required for vitamin K analysis. The Enviroprep Inert SPE cartridges, based on a glass/fluoropolymer composition, were found to have no fluorescence background for the conditions used in this assay. For the fractionation, the silica cartridge was eluted by gravity, providing a flow-rate of about 1 ml/min with hexane. The retention behavior of the vitamins K_1 and K_2 was determined by collection of 500-μl fractions, solvent exchange, and LC–fluorescence measurement. To determine the optimal fractionation conditions for the final method, crude extracts from a serum pool were selectively eluted with various eluent compositions and volumes. LC measurements were used to arrive at the protocol that provided optimal fractionation selectivity. Fats and less polar vitamins such as retinol are eluted with the initial volume of hexane. A more polar ether–hexane mixture eluted the K-vitamins. The concentration and volume of the ether–hexane eluent used was found to be crucial for a good separation of the K vitamins from interfering compounds. The more polar α- and γ-tocopherol elute in the fraction that immediately follows the K-vitamins and provide a serious interference in the LC–fluorescence determination. Thus, careful measurement of elution volumes is necessary to achieve selective and reproducible fractionation.

Fig. 4 shows the on-line catalytic reduction LC–fluorescence determination of serum vitamin

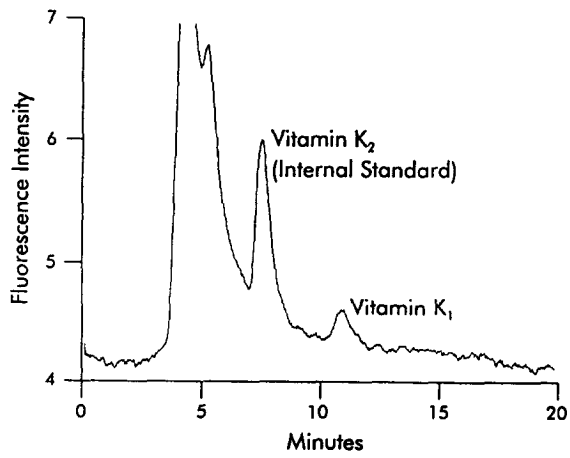


Fig. 4. Catalytic reduction/LC–fluorescence detection of vitamins K_1 and K_2 in a fractionated serum extract. Conditions: column, Vydac 201 TP 54 (0.46 × 25 cm, 5 μm); mobile phase, ethanol–methanol (40:60, v/v); flow-rate, 1.0 ml/min; detector, $\lambda_{ex} = 242$ nm, $\lambda_{em} = 430$ nm; sample injection, 14 pg vitamin K_1, 50 pg vitamin K_2.

K_1 using the xenon source detector. A serum volume of 500 μl was extracted and reconstituted to an equal volume. This sample was found to contain approximately 270 pg/ml of vitamin K_1. Note that even with the SPE fractionation, the elution of unresolved tocopherols can be seen as a large tailing peak immediately following the solvent front. With these separation conditions, a 20-min cycle time between injections was possible.

The recoveries of both spiked analyte (vitamin K_1) and internal standard (K_2) were determined using two serum pools. The overall recovery of extraction, fractionation, evaporation, and reconstitution steps is about 70 ± 34% (mean percent recovery ± 1 standard deviation, $n = 30$) for the vitamin K_2 internal standard. The low recovery and the high variance is likely to be the result of several factors. Perhaps most important are losses in the transfer of the small volumes of extract. Analyte losses may be particularly significant during the transfer of the evaporated crude extract into the SPE cartridge. In addition, redissolution of the extract following both of the evaporation steps may add to losses and to increase recovery variance. The evaporated crude extract has much fatty residue and must be carefully washed for good recovery. Even the evaporated, fractionated extract shows small amounts of fatty residue that appears to be partly insoluble in the final ethanolic solvent. Losses of the K-vitamins by photodegradation are also possible. Care was taken throughout the procedure to use amber vials when possible.

Accurate quantitation with the method relies on the similarity in the behavior (and losses) of the vitamins K_1 and K_2 throughout the procedure. Measurements were made on a single pooled serum to determine the method precision. A relative standard deviation of 32% ($n = 30$) was measured with a vitamin K_1 pool concentration of 420 pg/ml, which based on literature studies [3,10], should be a low-normal serum. Absolute verification of the method accuracy will require either comparison to established methods or agreement with the accepted value of a standard reference serum. Much of the measurement variance may be due to the inefficient manual sample handling, imprecise volume measurement in the fractionation and losses by decomposition. Laboratory robotic automation could help address some of the variance that results from these manipulative difficulties.

References

[1] M.F. Lefevere, A.P. De Leenheer and A.E. Claeys, J. Chromatogr., 186 (1979) 749–762.
[2] T. Ueno and J.W. Suttie, Anal. Biochem., 133 (1983) 62–67.
[3] W.E. Lambert, A.P. De Leenheer and E.J. Baert, Anal. Biochem., 158 (1986) 257–261.
[4] A.L.J.M. Pietersma-de Bruyn and P.M.M. van Haard, Clin. Chim. Acta, 150 (1985) 95–101.
[5] J.P. Langenberg and U.R. Tjaden, J. Chromatogr., 305 (1984) 61–72.
[6] P.M.M. van Haard, R. Engle and A.L.J.M. Pietersma-de Bruyn, Clin. Chim. Acta, 157 (1986) 221–230.
[7] T. Sakano, T. Nagakoka, A. Morimoto and K. Hirauchi, Chem. Pharm. Bull., 34 (1986) 4322–4326.
[8] K. Hirauchi, T. Sakano and A. Morimoto, Chem. Pharm. Bull., 34 (1986) 845–849.
[9] K. Hirauchi, T. Sakano, S. Notsumoto, T. Nagaoka, A. Morimoto, K. Fujimoto, S. Masuda and Y. Suzuki, J. Chromatogr., 497 (1989) 131–137.

[10] F. Moussa, L. Dufour, J.R. Didry, and P. Aymard, Clin. Chem., 35 (1989) 874–878.
[11] Y. Haroon, D.S. Bacon and J.A. Sadowski, Clin. Chem., 32 (1986) 1925–1929.
[12] M. Shino, Analyst, 113 (1988) 393–397.
[13] Y. Usui, N. Nishimura, N. Kobayashi, T. Okanoue, M. Kimoto and K. Ozawa, J. Chromatogr., 489 (1989) 291–301.
[14] M.F. Lefevere, R.W. Frei, A.H. Schotter and U.A. Th. Brinkman, Chromatographia, 15 (1982) 459–467.
[15] J.R. Poulsen and J.W. Birks, Anal. Chem., 61 (1989) 2267–2276.
[16] W.A. MacCrehan and W.E. May, in A.J. Dennis and M. Cooke (Editors), Proceedings of the 8th International Symposium on Polynuclear Aromatic Hydrocarbons, Batelle Press, Columbus, OH, 1984, 857–869.
[17] W.A. MacCrehan and W.E. May, Anal. Chem., 56 (1984) 625–628.
[18] W.A. MacCrehan, W.E. May, S.D. Yang, and B.A. Benner, Anal. Chem., 60, (1988) 194–199.
[19] W.A. MacCrehan, S.D. Yang and B.A. Benner, Anal. Chem., 60 (1988) 284–286.
[20] J.P. Langenberg and U.R. Tjaden, J. Chromatogr., 289 (1984) 377–385.