papers and notes on methodology

Multidimensional liquid chromatography: a breakthrough in the assessment of physiological vitamin K levels

M. F. Lefevere,* A. P. De Leenheer,**,[1] A. E. Claeys,* I. V. Claeys,*** and H. Steyaert****

Laboratorium voor Analytische Chemie and Laboratoria voor Medische Biochemie en Klinische Analyse,** Farm. Inst., Rijksuniversiteit Gent, Harelbekestraat 72, B-9000 Gent; Kliniek voor Inwendige Ziekten, Afd. Endocrinologie,*** A. Z., De Pintelaan 135, B-9000 Gent; and Seminarie voor Waarschijnlijkheidsrekening en Mathematische Statistiek,**** Rijksuniversiteit Gent, Krijgslaan 271, B-9000 Gent, Belgium*

Summary Owing to the lack of sensitivity and/or selectivity of the existing chemical assays, vitamin K deficiency has always been diagnosed indirectly by measuring its effect on blood coagulation. We used our recently developed multidimensional liquid chromatographic assay for what is, to the best of our knowledge, the first systematic investigation of physiological vitamin K levels in human blood. It allowed the unequivocal demonstration of *trans*-phylloquinone (vitamin K_1) and its quantification down to a level of 0.5 nanogram per milliliter of serum (ng/ml). In healthy adults, a mean serum concentration of 2.6 ng/ml was found, with a normal range of 0.9 to 7.8 ng/ml. These values apparently are distributed in a log-normal way.—**Lefevere, M. F., A. P. De Leenheer, A. E. Claeys, I. V. Claeys, and H. Steyaert.** Multidimensional liquid chromatography: a breakthrough in the assessment of physiological vitamin K levels. *J. Lipid Res.* 1982. **23:** 1068–1072.

Supplementary key words phylloquinone • serum • adsorption chromatography • reversed phase chromatography

In a previous article (1), we reported the development of a multidimensional LC procedure for the determination of phylloquinone (PK) in clinical serum samples. Since then, the method has been further elaborated and adapted to the handling of larger numbers of serum samples by adding an on-line sample clean-up step. This report describes the complete procedure, and its use in the positive identification and quantitative determination of the biologically active (2) *trans*-isomer of PK in serum.

MATERIALS AND METHODS

Chemicals

All reagents were analytical grade Merck or Baker analyzed, with the exception of *n*-hexane which was HPLC quality, and the liquid scintillation cocktail (Liploma®), which was purchased from Lumac (Basle, Switzerland). Phylloquinone (PK) was obtained from ICN-Pharmaceuticals (Cleveland, OH). The radiolabeled [1,2-$^3H_2$-phytyl]phylloquinone was a gift from Dr. Weber and Dr. Gloor (F. Hoffmann-La Roche & Co., Ltd., Basle, Switzerland), as were the two synthetic PK homologs, vitamin $K_{1(15)}$ and $K_{1(25)}$. All three were a mixture of *cis*- and *trans*-isomers, which were separated by preparative adsorption LC (1). The concentration of these standard solutions was determined by UV spectrophotometry at 248 and 270 nm. All were stored in the dark at 5°C.

Subjects

Serum samples were obtained from 40 apparently healthy fasting volunteers with no known vitamin deficiencies or medication. After centrifugation, the sera were immediately frozen and stored at −15°C until analyzed.

Extraction

To 2 ml of serum, 1500 dpm (±1 ng) of [^{3}H]-PK was added as internal standard in 20 μl of ethanol. After thorough mixing, the serum was deproteinized with 4 ml of ethanol and extracted once with 5 ml of *n*-hexane on a rotative extractor (Cenco Instr., Breda, Netherlands). The *n*-hexane layer was concentrated (Büchler Evapo-Mix, Büchler Inst. Inc., Fort Lee, NJ), reconstituted in 100 μl of *n*-hexane, to which *trans*-$K_{1(15)}$ and *trans*-$K_{1(25)}$ were added as markers, and injected entirely on the first chromatographic system. Isomerization and/or photodegradation of PK was avoided by working in the absence of daylight and by using low actinic (brown) glassware.

Liquid chromatographic analysis

trans-PK was resolved from co-extracted lipids by successive adsorption chromatography on underivatized silica (system 1) and reversed-phase (RP) chromatography (system 2) (**Fig. 1**). For system 1, a Hewlett-Packard HP 1084 A liquid chromatograph, equipped with a pneumatic column switching system (Valco Instr. Co., Houston, TX) was used. Upon the injection of the

Abbreviations: PK, phylloquinone (vitamin K_1); MK-n, menaquinone (vitamin K_2); LC, liquid chromatography (HPLC); GLC, gas–liquid chromatography; UV, ultraviolet; E.I., electron impact (mass spectrum); RP, reversed phase; ECD, electron capture detection.

[1] To whom all correspondence should be addressed.

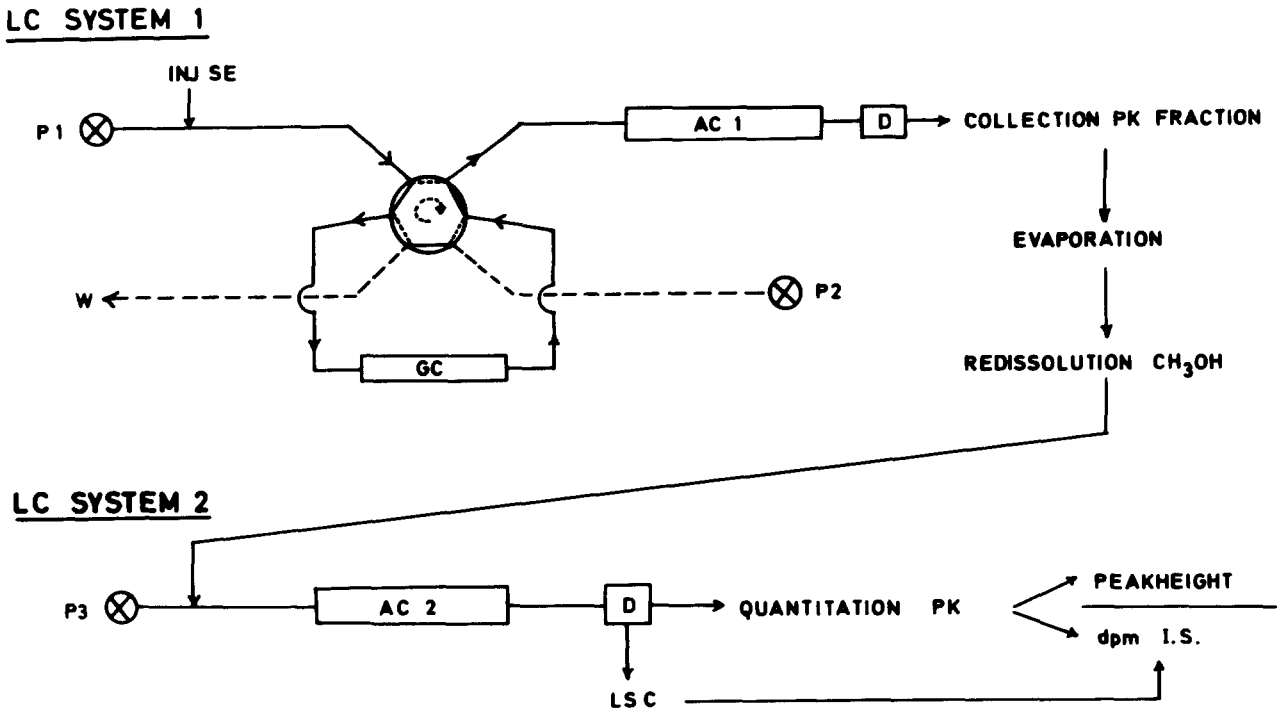

Fig. 1. Analytical scheme of multidimensional PK assay. PK is separated from the bulk of co-extracted lipids by adsorption chromatography (system 1) and then further resolved from co-eluting compounds on a reversed-phase system (system 2). More polar constituents of the serum extract (SE) are retained on a guard column (GC) and backflushed afterwards (W). Legend: P1 = pump 1, 3% diisopropylether in *n*-hexane; P2 = pump 2, backflush eluent (0.5% of methanol in *n*-hexane); GC = 10 cm × 4.6 mm ID Perisorb A (14 m^2/g) guard column; AC 1 = 20 cm × 7.0 mm ID silica column (Rsil 5 μm, 550 m^2/g); P3 = pump 3, 100% methanol; AC 2 = 15 cm × 3.2. mm ID RP 18 LL column; D = UV detector; LSC = liquid scintillation counting.

serum extract, eluent 1 (3% diisopropyl ether in *n*-hexane) was passed through the guard column (10 cm × 4.6 mm ID Perisorb A, E. Merck, Darmstadt, G.F.R., specific surface 14 m^2/g) until PK and the other lipophilic serum constituents eluted onto the silica fractionation column (20 cm × 7.0 mm ID totally porous Rsil 5 μm, Alltech Europe, 550 m^2/g). As soon as they arrived there (0.8 min), the valve was switched and the guard column was backflushed (eluted in reverse sense) with 0.5% of methanol in *n*-hexane, while *trans*-PK was separated from the bulk of lipophilic serum constituents on the fractionation column with the original eluent. A narrow fraction of this eluent, corresponding to the elution time of *trans*-PK was collected, and, after evaporation and reconstitution in methanol (40 μl), was rechromatographed on a non-fully capped, low load RP 18 column (1) using methanol as an eluent (1 ml/min). For this second system, a Spectra-Physics SP 8700 solvent delivery system equipped with a 20 μl loop injector (Rheodyne, Berkeley, CA) and a Pye Unicam LC3 variable-wavelength UV detector (Pye, Cambridge, U.K.) set at 248 nm (0.005 a.u.f.s.) were used. The amount of internal standard recovered throughout the whole procedure was determined by liquid scintillation counting (LSC) of the eluate of this second system (Packard 3380 Tricarb liquid scintillation spectrometer).

Calibration

Calibration was performed by analyzing 2-ml aliquots of a serum pool, supplemented with known amounts of *trans*-PK (2, 4, 6, 8, and 10 ng/ml).

Peak identification

The identity of the putative PK peak obtained in the second (RP) chromatogram, was verified by injecting a fraction of the corresponding eluate into a HP 5840 A gas chromatograph equipped with a ^{63}Ni electron capture detector and a short (1.2 m) 2% OV-101 column (260°C). We also ran a direct-inlet mass spectrum (LKB 9000 S, 20 eV, E.I.) on the pooled *trans*-PK peaks from 38 serum extracts, and compared it with the spectrum of an equal amount (± 100 ng) of PK standard. Of course, these experiments were performed without the addition of ^3H-labeled PK as internal standard.

RESULTS AND DISCUSSION

Extraction

On the rotative type extractor, higher extraction recoveries were obtained after deproteinization with ethanol (92–94%) compared to methanol (82–92%). A

similar observation was made by Abe et al. (3). After concentration of the extracts under reduced pressure, the last bit of *n*-hexane had to be removed in a nitrogen stream to avoid losses of PK under vacuum.

Liquid chromatographic analysis

The introduction of an on-line clean-up step with backflushing of the guard column considerably increased the lifetime of the analytical silica column. It also reduced the analysis time and prevented contamination of the PK fraction by late eluting peaks from previous injections. Inasmuch as the time of column-switching between guard and analytical column was chosen such that no compounds eluting after the MK fraction reached the analytical column, new injections could be made every 15 min (**Fig. 2A**). The analytical column lasted several months without regeneration.

In spite of these improvements and the use of a more selective solvent system (3% diisopropylether in *n*-hexane), compared to the original procedure (1), the first LC system did not succeed in the complete separation of *trans*-PK from UV-absorbing interferences. The collection of the fraction of the eluent containing *trans*-PK was facilitated by the use of *trans*-$K_{1(25)}$ and *trans*-$K_{1(15)}$, which elute, respectively, just in front and just after the *trans*-PK peak, as "markers". This fraction was rechromatographed then on the low load RP 18 column of system 2, which has a completely different selectivity and separated *trans*-PK well from the remaining UV absorbing compounds (Fig. 2B). The use of a small internal diameter column (4) for this step resulted in a detection limit of 400–550 pg.

Validation of the assay

The selectivity and specificity of the assay were confirmed by UV spectrophotometry and rechromatography of the putative *trans*-PK peak as previously described (1). Further evidence was gained from GLC–ECD chromatography and by direct-inlet E.I. mass spectrometry (**Fig. 3**). The molecular ion (m/z = 450) and three characteristic fragment ions (m/z = 225, 198 and 186) unequivocally demonstrated its identity.

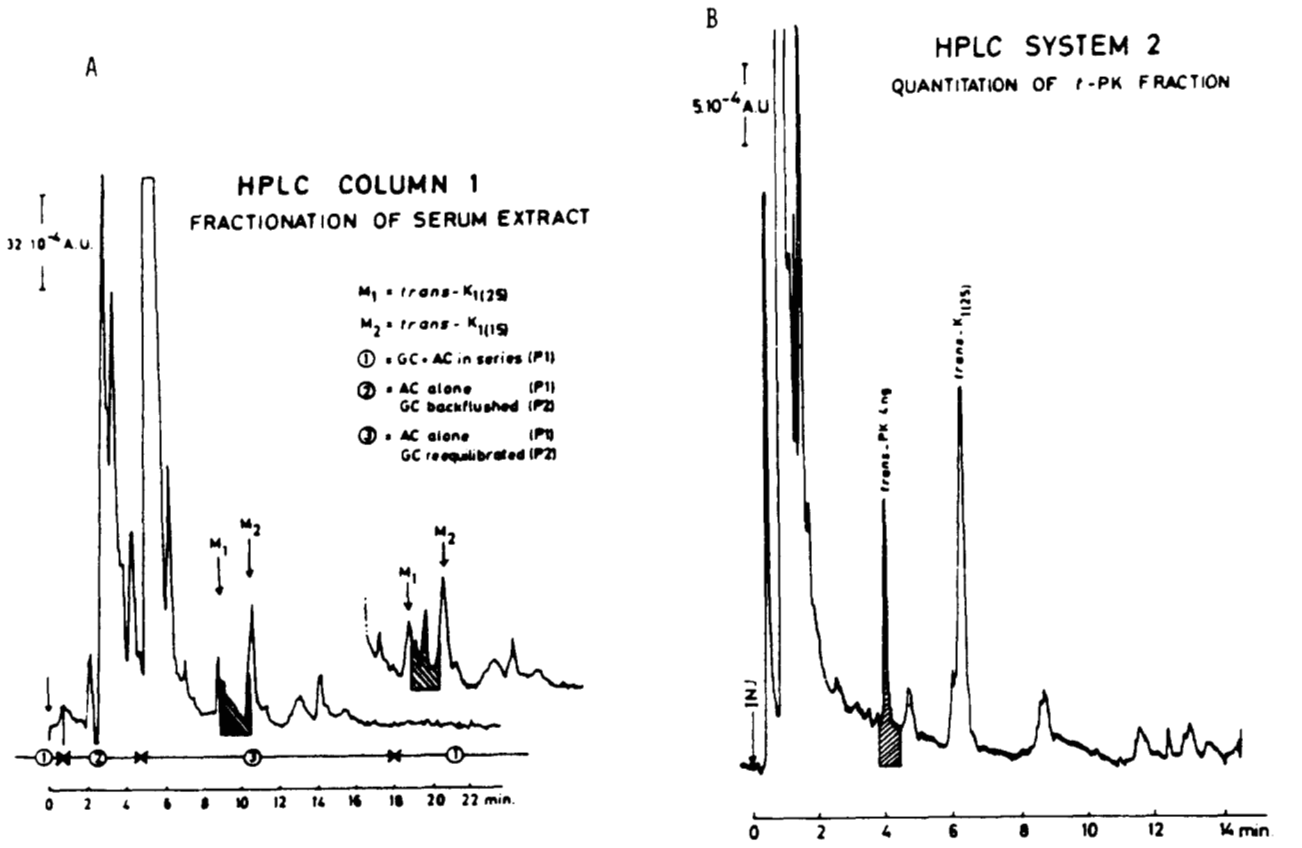

Fig. 2. A: LC step 1, fractionation of serum extract by adsorption chromatography on silica. (▬), Indicates the fraction containing *trans*-PK, which is collected for rechromatography on LC system 2. The injected amount corresponds to 2 ml of serum; detection: UV 254 nm. M1 and M2 indicate the two markers *trans*-$K_{1(25)}$ and *trans*-$K_{1(15)}$. Upper trace: serum extract of patient treated with PK. Also shown is the column switching sequence ①, ②, ③. Experimental conditions are as described for Fig. 1. B: LC step 2, quantification of *trans*-PK fraction by reversed phase (RP) chromatography; UV detection 248 nm. Injected fraction corresponds to 1 ml of serum. (▬), Indicates PK peak collected for liquid scintillation counting of internal standard. Experimental conditions are as described for Fig. 1.

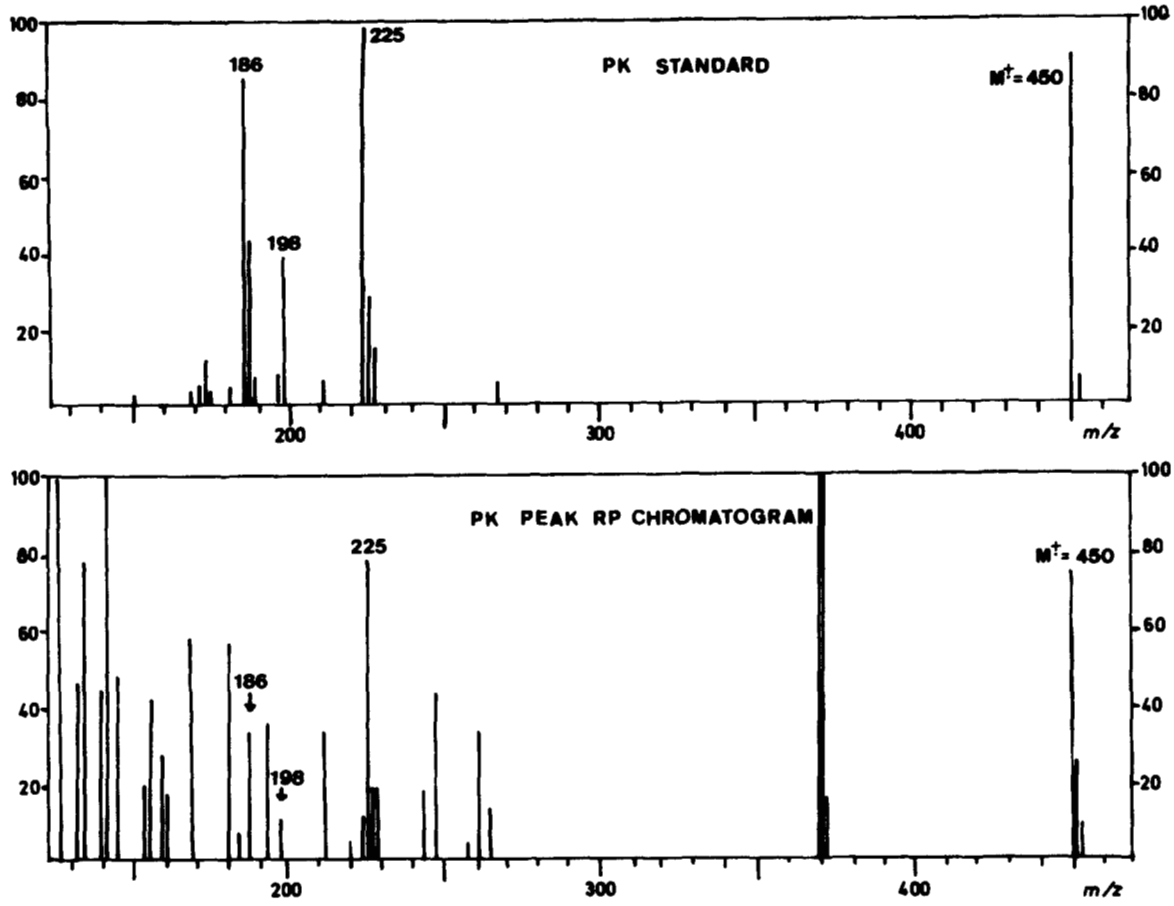

Fig. 3. Direct inlet, E.I. mass spectra of the pooled *trans*-PK peaks from 38 repetitive injections (no internal standard added), and from an equal amount (±100 ng) of *trans*-PK standard.

As to the quantitative aspect of the assay, a linear relationship was obtained between the ratio of the peak-height of the *trans*-PK peak (at 248 nm) to the corresponding radioactivity in the eluate (internal standard), and the amount of *trans*-PK in serum (r = 0.9973). In a typical experiment, we obtained a within-day precision of 5.3% (CV; n = 6; $\bar{x}$ = 5.5 ng/ml).

Physiological serum concentrations

Serum levels of *trans*-PK in 40 healthy adult fasting persons ranged from 0.8 to 8.7 ng/ml. The apparent log-normal distribution of these values (**Fig. 4**) was confirmed by a Kolmogorov-Smirnov test (5, 6) at a level of significance of 0.05. A similar phenomenon has been observed for trace elements and a number of other blood constituents, as well as for liver reserves of vitamin A (7–11). On this basis, the mean serum concentration of *trans*-PK was estimated at 2.6 ng/ml with a normal range of 0.9 to 7.8 ng/ml [$\overline{\log x} \pm 2$ S.D.$(\log x)$]. Ninety-five percent of the serum concentrations found was within the limits of this normal range.

Up to now, we have confined ourselves to the measurement of *trans*-PK, which probably is the most important individual nutritional source of vitamin K to humans. The same fractionation and purification procedure has been used also to isolate the menaquinone (MK-n) fraction of serum, but in contrast to liver ex-

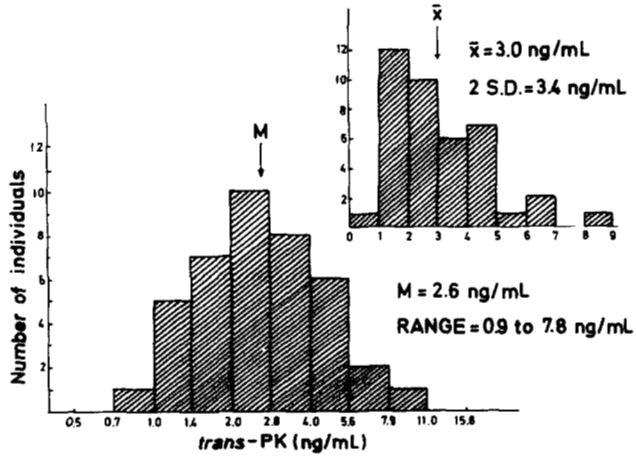

Fig. 4. Frequency distribution curves of *trans*-PK levels found in the sera of 40 normal, healthy adults. Upper trace: arithmetic plot; lower trace: logarithmic scale, data grouped in equidistant cell widths. (M) = average serum concentration.

tracts (12, 13), none of these could be identified in serum until now. A similar observation was made by Chiu, Zee-Cheng, and Olson (14).

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