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Diet-sensitive prognostic markers for cardiovascular and renal disease

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Chapter 7

Measurement of Plasma Vitamin K₁ (Phylloquinone) and K₂ (Menaquinones-4 and -7) using HPLC-Tandem Mass Spectrometry

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Abstract

Background. Given the growing interest in the health benefits of vitamin K, there is great need for development of new high-throughput methods for quantitative determination of vitamin K in plasma. We describe a simple and rapid method for measurement of plasma vitamin K₁ (phylloquinone [PK]) and K₂ (menaquinones [MK]-4 and -7). Furthermore, we investigated the association of fasting plasma vitamin K with functional vitamin K insufficiency in renal transplant recipients (RTR).

Methods. We used HPLC-tandem mass spectrometry with atmospheric pressure chemical ionization for measurement of plasma PK, MK-4, and MK-7. Solid-phase extraction was used for sample clean-up. Mass spectrometric detection was performed in multiple reaction monitoring mode. Functional vitamin K insufficiency was defined as plasma desphospho-uncarboxylated matrix Gla protein (dp-ucMGP) >500 pmol/L.

Results. Lower limits of quantitation were 0.14 nmol/L for PK and MK-4 and 4.40 nmol/L for MK-7. Linearity up to 15 nmol/L was excellent. Mean recoveries were >92%. Fasting plasma PK concentration was associated with recent PK intake ($\rho=0.41$, $P=0.002$) and with plasma MK-4 ($\rho=0.49$, $P<0.001$). Plasma PK ($\rho=0.38$, $P=0.003$) and MK-4 ($\rho=0.46$, $P<0.001$) were strongly correlated with plasma triglyceride concentrations. Furthermore, we found that MK-4-triglyceride ratio, but not PK-triglyceride ratio, was significantly associated with functional vitamin K insufficiency (OR 0.22 [0.07-0.70], $P=0.01$) in RTR.

Conclusions. The developed rapid and easy-to-use LC-MS/MS method for quantitative determination of PK, MK-4, and MK-7 in human plasma may be a good alternative for the labor-intensive and time-consuming LC-MS/MS methods and enables a higher sample throughput.

Introduction

There is increasing interest in the potential health benefits of vitamin K (1). Several studies have reported a role for vitamin K in bone health (2,3), vascular calcification (3), and cardiovascular risk (1,4,5). The main dietary form of vitamin K in the Western diet is vitamin K₁, or phylloquinone (PK), which is mainly found in green vegetables (1,6). Vitamin K₂, or menaquinone (MK-*n*), is mainly from microbial origin and is found in fermented foods as cheese, curd, and natto (1). Vitamin K₃ (menadione) is a synthetic form of vitamin K present in animal feeds (1).

Vitamin K status can be assessed by functional assays such as prothrombin time, which lacks sensitivity to detect subclinical vitamin K insufficiency (7,8), or by measurement of undercarboxylated proteins as osteocalcin (ucOC) and matrix Gla protein (ucMGP), which are more sensitive to detect subclinical vitamin K insufficiency. Direct quantification of vitamin K in plasma, however, was reported to be the best indicator of recent dietary intake (9-11).

Several methods for direct quantification of vitamin K in plasma have been described. Most methods use high-performance liquid chromatography (HPLC) with fluorescence detection after postcolumn reduction (12-14), which requires extensive sample prepurification to decrease chromatographic interference from co-extracted lipids (8). More recently, three methods for determination of plasma vitamin K were developed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) (8,15,16). Unfortunately, sample prepurification procedures remain labor-intensive and total analyses-times long. The development of new LC-MS/MS methods can be useful for speeding-up the sample preparation process and obtaining a higher sample throughput. In this study, we describe a simple and rapid LC-MS/MS method for determination of PK, MK-4, and MK-7 in human plasma. Furthermore, we investigated the association of plasma vitamin K concentration with vitamin K intake and functional vitamin K status, as derived from plasma desphospho-uncarboxylated matrix Gla protein (dp-ucMGP), in renal transplant recipients (RTR).

Materials and Methods

Reagents. We obtained LC-MS-grade methanol, acetonitrile, isopropanol, AR-grade ethanol, HPLC-grade hexane, and UPLC-MS-grade formic acid from Biosolve BV. PK and MK-4 were purchased from Supelco (Sigma-Aldrich), MK-7 from Toronto Research Chemicals Inc. The D₇-labeled internal standards (ISs) for PK, MK-4, and MK-7 were purchased from IsoSciences, Syncom, and Toronto Research Chemicals Inc., respectively.

Stock solutions, calibration standards, and quality controls. Individual stock solutions of the analytes were prepared dissolving the analytes and ISs in ethanol to form calibration standards. Pooled plasma was spiked with calibration standards to obtain low, medium, and high quality-control samples (see Table 2 for concentrations). Pooled plasma was diluted with phosphate buffered saline (PBS) before enrichment with PK, MK-4, and MK-7 to obtain low quality-control samples. Amber colored vials were used to avoid photodegradation of vitamin K.

Instrumentation. The HPLC analyses were conducted with a Shimadzu Prominence HPLC system consisting of a parallel double binary pump (LC-20AD), system controller (CBM-20A), automatic solvent degasser (DGU-20A5), and autosampler (SIL-20A HT). The autosampler temperature was maintained at 15 °C. HPLC was performed using a Phenyl-Hexyl column (particle size 3 µm, 2.0 mm internal diameter by 100 mm; Phenomenex). Column temperature was controlled with a column oven (CTO-20AC). Mass spectrometry was performed with an AB SCIEX Triple Quad 5500 System equipped with an atmospheric pressure chemical ionization (APCI) ion source.

Liquid chromatography conditions. The binary gradient system consisted of methanol/water (1:1) acidified with formic acid (eluent A) and methanol acidified with formic acid (eluent B). Gradient elution was applied as follows: 0-1 min, 80% A, 20% B; 1-5 min, convex curve gradient, 100% B; 5-12 min, 100% B; reequilibration from 12 to 14 min with 80% A, 20% B. A gradient in flow rate was applied as follows: 0-3 min, 0.4 mL/min; 3-5 min, linear gradient, 0.7 mL/min; 5-10 min, 0.7 mL/min; 10-10.50 min, linear gradient, 0.4 mL/min; 10.50-14 min, reequilibration with 0.4 mL/min. Column temperature was kept constant at 50°C.

Mass spectrometry conditions. The APCI ion source operated in positive ion mode with a nebulizer current of 3 µA and a probe temperature of 400 °C. Ultrahigh-purity nitrogen was used as curtain gas (35 psi) and collision gas (setting 8), zero air was used as nebulizer gas (60 psi). Identification and quantification were based on MS/MS multiple reaction monitoring (MRM). An overview of the MRM transitions, collision energies, and retention time for the analytes are given in Table 1. All aspects of system

operation and data acquisition were controlled using Analyst 1.5.1. software.

Sample preparation. Blood samples were collected by venipuncture in 5 mL Vacutainer Tubes (Becton Dickinson) containing Na₂EDTA as anticoagulant. After centrifugation (4 °C, 3500 rpm [2500 g], 10 min), plasma was transferred to glass tubes and stored at -20 °C until analysis. Before analysis, 350 µL aliquots of plasma were transferred into 2 mL amber colored Sarstedt tubes and spiked with 50 µL of IS. Ethanol (175 µL) was added to denature the proteins. After mixing for 2 min, the solution was centrifuged at 3500 rpm for 5 min. The supernatant was applied to Oasis® HLB solid-phase extraction (SPE) cartridges (1cc/30 mg sorbent; Waters), which were washed with 1 mL of methanol/water (1:9) with 1% formic acid and 1 mL acetonitril/water (8:2) with 1% formic acid and eluted with 2 mL methanol/isopropanol/hexane (2:1:1). The eluate was evaporated under nitrogen. The residue was reconstituted in 150 µL acetonitrile with 1% formic acid and vortexed for 10 s. The solutions were transferred to (amber) microvials, capped, and placed in an autosampler rack. Aliquots of 25 µL were automatically injected into the HPLC system.

Table 1. LC-MS/MS parameters.

	Precursor ion (m/z)	Collision energy (eV)	Product ion (m/z)	Retention time (min)
Analytes				
PK	451.3	35	187.1	6.09
MK-4	445.3	35	187.1	5.96
MK-7	649.5	42	187.1	6.88
Internal standards				
PK-d7	458.3	35	194.1	6.08
MK-4-d7	452.3	35	194.1	5.95
MK-7-d7	656.5	42	194.1	6.87

Abbreviations: MK-4, menaquinone-4; MK-7, menaquinone-7; PK, phylloquinone.

Method validation

The LC-MS/MS method for analysis of PK, MK-4, and MK-7 in human plasma was validated according to internal policy in compliance with CLSI and ISO 15189 guidelines.

Quantitation limits. Lower limits of quantitation (LLOQ) were determined from 6 replicates by injecting serially diluted plasma samples containing PK, MK-4, and MK-7 using control human plasma. LLOQ was defined as the concentration at which the coefficient of variation was $\leq 20\%$.

Carryover. Carryover was estimated by alternating injections of plasma samples spiked with high and low concentrations of PK, MK-4, and MK-7.

Linearity. Calibration curves were established by plotting the peak area ratios (analyte/IS) against PK, MK-4, and MK-7 at 8 concentrations over the range of 0.8 to 15.5 nmol/L for PK, 0.7 to 14.2 nmol/L for MK-4, and 0.8 to 15.7 nmol/L for MK-7. The regression equations of the calibration curves were calculated using MS Excel. The dilutional linearity of the assay was performed in duplicate by serial dilution of samples containing high concentrations of the analytes with PBS.

Precision. Intra- and interassay variation were determined using quality-control samples with the analytes in low, medium, and high concentrations. Intra-assay precision was obtained from 10 replicates measured in a single series and interassay imprecision from at least 10 different assays over a period of 3 weeks.

Recovery. Mean relative recoveries were estimated by addition of PK, MK-4, and MK-7 in low (3.5 nmol/L), medium (7.5 nmol/L), and high (15 nmol/L) concentrations to pooled plasma. Recoveries were measured in three replicate samples in two series.

Matrix effect. Matrix effects were estimated by diluting (0%, 25%, 50%, and 75%) samples spiked with 15 nmol/L PK, MK-4, and MK-7 with samples low in PK, MK-4, and MK-7 and by injection of a sample low in PK, MK-4, and MK-7 while infusing a standard solution containing PK, MK-4, and MK-7 to the mass spectrometer interface.

Stability. Samples with medium (7.5 nmol/L) and high (15 nmol/L) concentrations of PK, MK-4, and MK-7 were measured in duplicate after different storage conditions. Samples were stored in amber colored vials, unless stated otherwise. The 1st set was measured immediately and served as reference point; other sets were stored at 4 °C for 24 h, 72 h, and 7 days; at room temperature for 24 and 72 h (stored in the dark); at room temperature for 2, 4, 8, and 24 h (stored in transparent tubes in artificial light); frozen at -20 °C for 24 h, 72 h, 6 weeks, and 12 weeks. Autosampler stability was assessed at 24 h, 72 h, 7 days, and 14 days. Remaining samples were frozen at -20 °C and stability was investigated after 1 to 3 freeze-thaw cycles.

Clinical applications

Study population. We measured plasma vitamin K in a cohort of 60 RTR (50% male; age 55 ± 10 years; eGFR 48 ± 16 mL/min/1.73 m²) (17,18). RTR attending the outpatient clinic of the UMCG aged ≥ 18 years and able to return completed food records were eligible for inclusion. Exclusion criteria were known malignancy, abnormal liver function tests, history of gastrointestinal disease or metabolic disease, or active infection (17). The institutional review board approved the study protocol (METc 2008/186), which adhered to the Declaration of Helsinki.

Measurements. Dietary vitamin K intake was assessed using a dietary diary that was kept during three consecutive days in advance of the patients' visit to the outpatient clinic (17). All patients adhered to their normal dietary habits. A trained researcher checked whether diaries were filled out properly, and if necessary additional information was obtained about unusual or missing reports. For calculations of the intakes of total energy and nutrients, we used the Food Calculation System (BAS nutrition software 2004, Arnhem, the Netherlands) in which Dutch food composition database NEVO 2006 was included (19). Concentrations of vitamin K₁ and K₂ (MK-4 through MK-10) of 260 foods have been added to the NEVO 2006 food database, as described previously (4).

Blood was drawn after an 8-12 h overnight fasting period. The creatinine-based Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation was used to estimate glomerular filtration rate (GFR) (20). Plasma dp-ucMGP levels were measured in citrated plasma using a dual-antibody ELISA (VitaK Maastricht, the Netherlands) (17). Plasma dp-ucMGP concentrations were found to be particularly sensitive for changes in vascular vitamin K status (21). Therefore, in line with previous studies (17,18), functional vitamin K insufficiency was defined as dp-ucMGP >500 pmol/L (21).

Statistical analyses

Statistical analyses were performed using SPSS version 22.0 for Windows (IBM Corporation, Chicago, IL). Results were expressed as mean \pm standard deviation (SD) for normally distributed data, median [interquartile range] for non-normally distributed data, and number of patients (percentage) for nominal data. A two-sided $P < 0.05$ was considered to indicate statistical significance.

The residuals method was used to calculate energy-adjusted vitamin K intake (22). Vitamin K intake data were logarithmically transformed to fulfill criteria for linear regression analyses. We used Spearman's correlation coefficients to investigate associations between plasma vitamin K, vitamin K intake, and other clinical parameters. The Mann Whitney U test was used to compare plasma vitamin K-triglyceride ratio's

among subjects with and without functional vitamin K insufficiency. Logistic regression analyses were used to assess whether plasma vitamin K and vitamin K intake were associated with functional vitamin K insufficiency. Given the effects of vitamin K antagonists on dp-ucMPG levels (17), we excluded RTR who used vitamin K antagonists (n=6) from logistic regression analyses. In secondary analyses, we investigated the association of energy-adjusted vitamin K intake with functional vitamin K insufficiency.

Results

Method validation

Chromatography. Chromatograms of PK, MK-4, and MK-7 obtained by LC-MS/MS in MRM are shown in Figure 1.

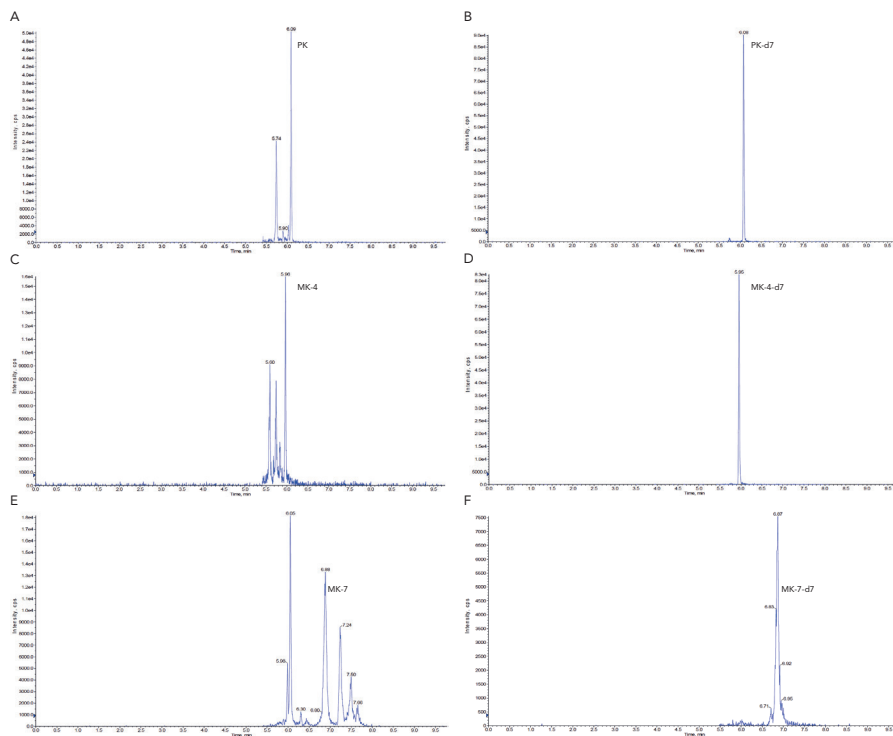


Figure 1. Chromatograms of plasma PK (3.1 nmol/L), MK-4 (2.8 nmol/L), and MK-7 (15.7 nmol/L) and their deuterated (d7) internal standards obtained by LC-MS/MS in MRM mode.

Quantitation limits. LLOQ was 0.14 nmol/L for PK, 0.14 nmol/L for MK-4, and 4.40 nmol/L for MK-7.

Carryover. Carryover was <0.1% between alternating injections of plasma samples with high and low concentrations of PK, MK-4, and MK-7.

Linearity. Linearity over the calibration ranges of all three analytes was excellent, with mean correlation coefficients (R^2) of 0.998 for PK, 0.999 for MK-4, and 0.994 for MK-7. Plasma samples with high concentrations of PK, MK-4, and MK-7 that exceed the calibration range can be diluted up to 10 times.

Precision. Results on intra- and interassay variation are shown in Table 2. Mean intra- and interassay CV's for PK were <10%. Intra- and interassay CV's were <10% for MK-4 concentrations ≥ 4 nmol/L. Concentrations of MK-7 in low and medium quality-control samples were <LLOQ and could not be reported in a quantitative manner. Intra- and interassay CV's for MK-7 concentrations ≥ 8 nmol/L were <12%.

Recovery. Mean recoveries are shown in Table 2. Overall, mean recovery was 92% for PK, 103% for MK-4, and 99% for MK-7.

Table 2. Intra- and interassay variation, and recovery of the LC-MS/MS method for plasma PK, MK-4, and MK-7.

	Intraassay ^a			Interassay ^b			Recovery
	Mean (nmol/L)	SD (nmol/L)	CV (%)	Mean (nmol/L)	SD (nmol/L)	CV (%)	Mean (range) (%)
PK							
Low	0.41	0.03	8.4	0.40	0.04	9.8	91.4 (80.5-100.4)
Medium	2.44	0.17	7.0	2.37	0.20	8.6	92.3 (84.5-101.0)
High	9.05	0.38	4.2	8.31	0.75	9.1	93.0 (85.3-99.5)
MK-4							
Low	0.20	0.03	14.1	0.17	0.03	16.2	98.0 (91.9-100.8)
Medium	0.40	0.07	16.7	0.35	0.05	14.6	105.1 (101.4-107.5)
High	3.95	0.17	4.3	4.18	0.26	6.2	105.3 (103.1-109.0)
MK-7							
High	7.98	0.88	11.0	12.25	1.37	11.2	98.6 (92.1-104.7)

Abbreviations: CV, coefficient of variation; MK-4, menaquinone-4; MK-7, menaquinone-7; PK, phylloquinone; SD, standard deviation.

^a n=10; ^b n=25 for PK and MK-4, n=9 for MK-7.

Matrix effect. Dilution of samples with high concentrations of the analytes with pooled plasma resulted in a linear curve. A weak ion suppression effect was noted for PK and MK-4, but not for MK-7, after injection of a sample low in PK, MK-4, and MK-7 while infusing a standard solution containing the three analytes to the mass spectrometer interface (Figure 2).

Stability. PK, MK-4, and MK-7 were stable up to 7 days in plasma stored at 4°C and up to 14 days at 15°C (i.e., autosampler temperature). At room temperature, PK, MK-4, and MK-7 were stable up to 72 h when stored in the dark and up to 24 h when stored in

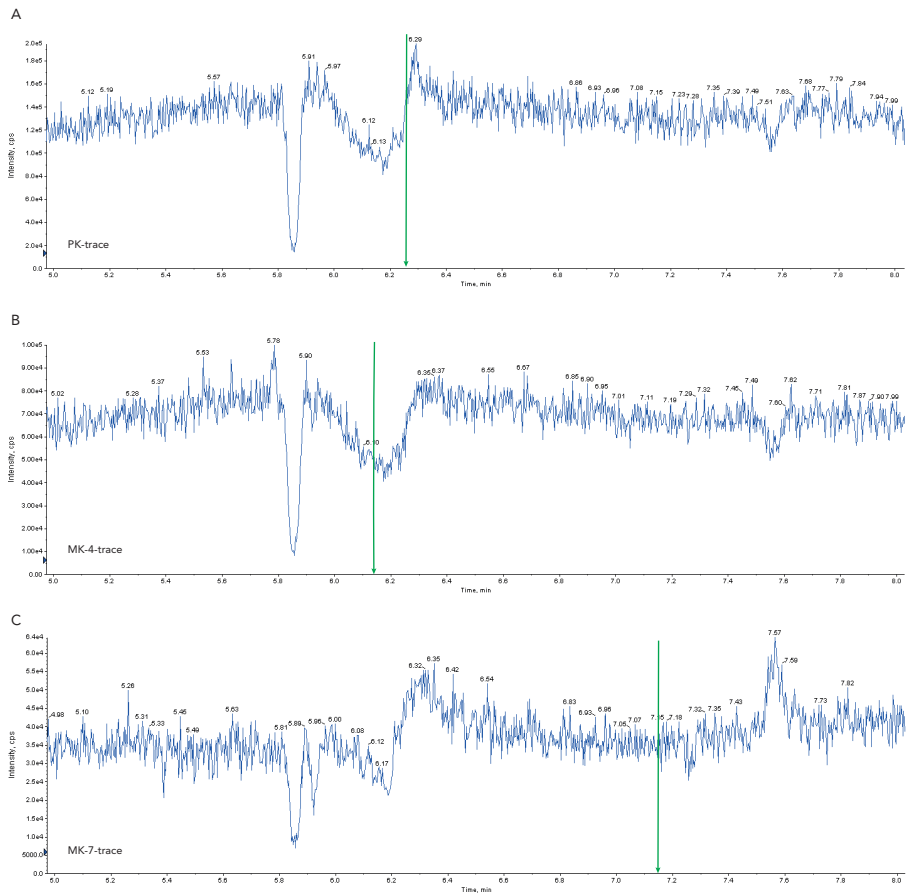


Figure 2. Post-column infusion chromatograms for plasma PK (A), MK-4 (B), and MK-7 (C) after injection of a plasma sample with low concentrations of PK, MK-4, and MK-7. The arrows in the graph represent the retention time of the analytes.

transparent tubes in artificial light. PK, MK-4, and MK-7 were stable up to 12 weeks at -20 °C. We found no significant changes in measured concentrations of PK, MK-4, and MK-7 after 1 to 3 freeze-thaw cycles.

Clinical applications

Data on vitamin K intake and status in this cohort of RTR are depicted in Table 3. None of the plasma PK levels were below the normal range reported in literature (i.e., 0.38-6.77 nmol/L (3)), whereas plasma MK-4 levels were below normal reference values (i.e., 0.16-6.01 nmol/L (3)) in 8 out of 60 (13%) RTR. Dietary vitamin K intake was below the recommended level in 41 out of 60 RTR (68%; Table 3). Forty-eight out of 60 (80%) RTR had functional vitamin K insufficiency (i.e., dp-ucMGP >500 pmol/L; Table 3).

Table 3. Plasma vitamin K, vitamin K intake, and functional vitamin K status in renal transplant recipients (n=60).

	Total cohort
Plasma vitamin K	
PK (nmol/L)	1.35 (0.89-2.32)
MK-4 (nmol/L)	0.20 (0.17-0.25)
MK-7 (nmol/L)	<4.40
Dietary intake^a	
Vitamin K ₁ (µg/day)	45.8 (16.7-128.4)
Vitamin K ₂ (µg/day)	12.6 (2.4-25.7)
Total vitamin K (µg/day)	63.3 (38.2-131.6)
Inadequate intake (n, %) ^b	41 (68)
Total energy intake (kcal/day)	1923 ± 512
Energy-adjusted vitamin K ₁ (µg/day) ^c	57.0 (3.6-908.3)
Energy-adjusted vitamin K ₂ (µg/day) ^c	6.7 (0.2-236.0)
Energy-adjusted total vitamin K (µg/day) ^c	85.2 (9.1-797.9)
Functional vitamin K status	
dp-ucMGP (pmol/L)	753 (543-1091)
Functional vitamin K insufficiency (n, %) ^d	48 (80)
Use of vitamin K antagonists (n, %)	6 (10)

^a Intake assessed by dietary diary on the last day before blood withdrawal.

^b Intake below recommended values: 90 µg/day (F), 120 µg/day (M) (23).

^c Geometric mean with 95% CI.

^d Dp-ucMGP >500 pmol/L (17,21).

Plasma PK was strongly correlated with PK intake on the last day before blood withdrawal ($\rho=0.41$; $P=0.002$) and with plasma MK-4 ($\rho=0.49$; $P<0.001$). Furthermore, both plasma PK and MK-4 were strongly correlated with plasma triglycerides ($\rho=0.38$, $P=0.003$ and $\rho=0.46$, $P<0.001$, respectively). Because of the strong correlation of plasma PK and MK-4 with triglycerides, we also calculated the ratio of PK and MK-4 to triglyceride concentration.

Although there was a stronger trend for a higher intake of vitamin K₂, rather than PK, to be associated with functional vitamin K insufficiency, these associations did not reach statistical significance (Table 4). The associations of energy-adjusted vitamin K intake with functional vitamin K insufficiency were essentially the same (data not shown). In univariable analyses, we found a significant association of plasma MK-4-triglyceride ratio, but not PK-triglyceride ratio, with functional vitamin K insufficiency. This association remained significant after adjustment for age and sex (Table 4). Plasma PK and MK-4-triglyceride ratio's for RTR with and without functional vitamin K insufficiency are depicted in Figure 3.

Table 4. Associations of plasma vitamin K and vitamin K intake with functional vitamin K insufficiency (dp-ucMGP >500 pmol/L) in renal transplant recipients who did not use vitamin K antagonists (n=54).

	Functional Vitamin K Insufficiency			
	Univariable		Multivariable	
	OR (95% CI)	P	OR (95% CI)	P
Plasma vitamin K				
PK-TG ratio (nmol/mmol)	0.91 (0.55-1.50)	0.7	0.85 (0.50-1.46)	0.6 ^b
MK-4-TG ratio (0.1 nmol/mmol)	0.22 (0.07-0.70)	0.01	0.16 (0.04-0.57)	0.005 ^b
Vitamin K intake^a				
Vitamin K ₁ (10 µg/day)	1.00 (0.98-1.08)	0.8	1.00 (0.98-1.02)	0.8 ^c
Vitamin K ₂ (10 µg/day)	0.77 (0.53-1.11)	0.2	0.72 (0.48-1.09)	0.1 ^c
Total vitamin K (10 µg/day)	1.00 (0.98-1.02)	0.9	1.00 (0.98-1.02)	0.9 ^c

^aVitamin K intake assessed by dietary diary on the last day before blood withdrawal.

^b Adjusted for age and sex.

^c Adjusted for age, sex, and triglycerides (TG).

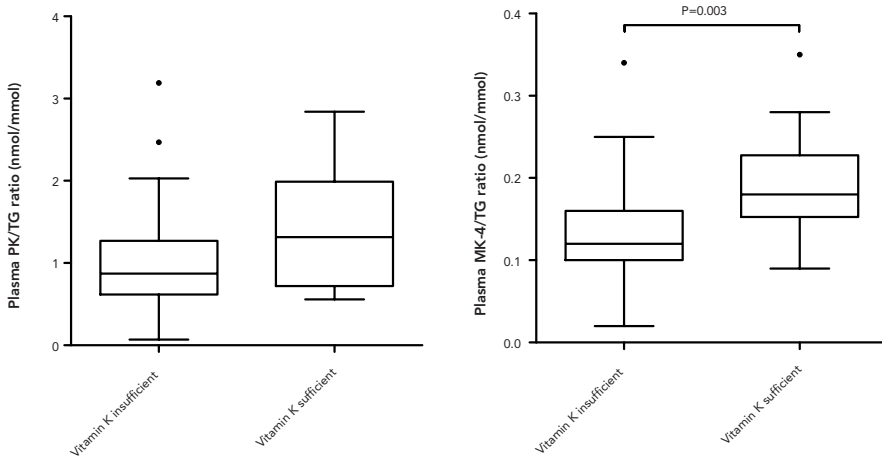


Figure 3. Plasma PK- and MK-4-triglyceride (TG) ratio's for renal transplant recipients with and without functional vitamin K insufficiency (i.e., dp-ucMGP >500 pmol/L).

Discussion

In this study, we describe a simple and rapid method for quantitative determination of PK, MK-4, and MK-7 in human plasma using LC-MS/MS. The simple sample preparation procedure avoids labor-intensive prepurification methods while the short total runtime enables a higher sample throughput. We found that plasma PK concentrations were significantly associated with recent PK intake and plasma MK-4 concentrations in RTR. Furthermore, plasma MK-4-triglyceride ratio, but not PK-triglyceride ratio, was significantly associated with functional vitamin K insufficiency in RTR.

Vitamin K is a fat-soluble vitamin essential for bone and vascular health (1,3,24,25). Given the growing interest in the health benefits of vitamin K, there is a need for a standardized method for measurement of plasma vitamin K that enables a higher sample throughput. Measurement of vitamin K in plasma, however, is an analytical challenge because of the low circulating vitamin K levels, the non-polar character of vitamin K, and interference of co-extracted lipids. The most commonly used method for measurement of plasma vitamin K is HPLC with fluorescence detection, which requires extensive sample prepurification to decrease chromatographic interference from co-extracted lipids (8). Suhara et al. were the first to develop an LC-APCI-MS/MS method for measurement of PK, MK-4, and MK-7 in plasma, offering much greater sensitivity and selectivity compared with other techniques (15). However, the sample prepurification procedure included a dual step extraction and chromatographic

separation was achieved in 80 min followed by a wash and reequilibration period of 40 min (15), which is a major drawback for applying this method in routine analysis. The sample prepurification process was simplified (i.e., single step LLE) and total run-time was reduced to ~20 min in more recently developed LC-APCI-MS/MS methods (8,16). However, these methods did not include MK-7 (8,16), and the sensitivity of the assay for MK-4 was insufficient to detect low plasma MK-4 levels (16). We further simplified the sample prepurification process and reduced the total run-time towards 14 min. In agreement with previously developed methods (8,15,16), our method showed excellent linearity and consistent recoveries. Our method allows reproducible quantification of plasma PK across a broad concentration range and at higher concentrations of MK-4 with intra- and interassay variation <10%. The latter are similar to CV's of previously developed LC-APCI-MS/MS methods. The LLOQ for MK-4 was similar to that of Suhara et al. (15) and lower compared with a recently developed method of Gentili et al. (16), allowing for quantification of lower circulating levels of MK-4.

In RTR, median plasma PK and MK-4 concentrations were 1.35 and 0.20 nmol/L, respectively. Fusaro et al. (3) found median PK, MK-4, and MK-7 concentrations of 1.40 nmol/L, 1.14 nmol/L, and 1.68 nmol/L, respectively, in hemodialysis patients. Suhara et al. (15) found mean PK, MK-4, and MK-7 concentrations of 2.71 nmol/L, 0.88 nmol/L, and 9.82 nmol/L, respectively, in healthy Japanese adults. In the Netherlands, the main dietary form of vitamin K is PK; an estimated 10-25% of dietary vitamin K is provided by menaquinones (1). Since plasma vitamin K levels are closely related to recent dietary intake of vitamin K (9-11), differences in vitamin K intake between the healthy Japanese adults, Italian dialysis patients, and Dutch RTR are likely to explain the differences in plasma vitamin K concentrations.

In line with previous studies (9-11), we found that plasma PK was associated with recent PK intake. Plasma MK-4, however, was not associated with dietary intake of vitamin K₂, but was associated with plasma PK concentration. A recent study in rodents showed that PK is converted into MK-4 via side chain removal-addition (26). Our data suggest that PK is also converted to MK-4 in humans. Furthermore, in line with previous studies (10,11,27), we found a strong correlation of fasting plasma vitamin K with triglycerides, which could be explained by the fact that PK is mainly transported by triglyceride-rich lipoproteins, in particular VLDL, in plasma (11,28,29). This finding underlines the importance of adjusting plasma vitamin K concentrations for triglyceride concentrations (11).

Several studies suggested that menaquinones may be more effective in activating extra-hepatic vitamin K-dependent proteins than PK (1). In this study, we found a stronger inverse trend of vitamin K₂, rather than vitamin K₁, intake with functional

vitamin K insufficiency, but these associations did not reach statistical significance. However, plasma MK-4–triglyceride ratio, but not PK–triglyceride ratio, was significantly associated with functional vitamin K insufficiency, supporting the hypothesis that vitamin K₂ may be more effective in activating extra-hepatic vitamin K-dependent proteins than PK.

Several limitations of the present study need to be addressed. Because we found only one intense product fragment of the vitamin K homologues, we used one transition for identification and quantification of vitamin K homologues with tandem mass spectrometry, which is in line with previous studies (8,15). In terms of specificity, it would be better to evaluate the relative intensities of mass transitions derived from at least two different fragments. In addition, the current LLOQ of 4.40 nmol/L for MK-7 is higher than desirable for studies in a Western population because intake of MK-7 is often very low, resulting in values below the LLOQ. Although intra- and interassay variation CV's for low concentrations of MK-4 and MK-7 were >15%, the intra- and interassay variation CV's for PK and for higher concentrations of MK-4 and MK-7 were <12%, meeting general bioanalytical method validation standards. Therefore, our method is suitable for measurement of PK and could be suitable for measurement of MK-4 and MK-7 in populations that consume higher amounts of MK-4 and MK-7 or for monitoring of treatment with MK-4 and MK-7.

Conclusions

We describe a simple and rapid method for quantitative determination of PK, MK-4, and MK-7 in human plasma using LC-MS/MS. Previous studies and functional markers like dp-ucMGP have indicated that vitamin K insufficiency is a common problem, particularly in chronic kidney disease, in which it may have important health consequences (3,17,18). Status markers like plasma levels of PK, MK-4, and MK-7, are likely to provide relevant additional information in this rapidly emerging field.

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