methods

Determination of the urinary aglycone metabolites of vitamin K by HPLC with redox-mode electrochemical detection

Dominic J. Harrington,[1,*] Robin Soper,[†] Christine Edwards,[†] Geoffrey F. Savidge,[*] Stephen J. Hodges,[§] and Martin J. Shearer[*]

Centre for Haemostasis and Thrombosis,[*] The Haemophilia Reference Centre, St. Thomas' Hospital, London, UK; Department of Biological Sciences,[†] University of Essex, Colchester, UK; and Institute of Hepatology,[§] Liver Failure Group, Department of Medicine, University College, London, UK

Abstract **We describe a method for the determination of the two major urinary metabolites of vitamin K as the methyl esters of their aglycone structures, 2-methyl-3-(3′-3′-carboxymethylpropyl)-1,4-naphthoquinone (5C-aglycone) and 2-methyl-3-(5′-carboxy-3′-methyl-2′-pentenyl)-1,4-naphthoquinone (7C-aglycone), by HPLC with electrochemical detection (ECD) in the redox mode. Urinary salts were removed by reversed-phase (C_{18}) solid-phase extraction (SPE), and the predominantly conjugated vitamin K metabolites were hydrolyzed with methanolic HCl. The resulting carboxylic acid aglycones were quantitatively methylated with diazomethane and fractionated by normal-phase (silica) SPE. Final analysis was by reversed-phase (C_{18}) HPLC with a methanol-aqueous mobile phase. Metabolites were detected by amperometric, oxidative ECD of their quinol forms, which were generated by postcolumn coulometric reduction at an upstream electrode. The assay gave excellent linearity (typically, $r^2 \geq 0.999$) and high sensitivity with an on-column detection limit of <3.5 fmol (<1 pg). The interassay precision was typically 10%. Metabolite recovery was compared with that of an internal standard [2-methyl-3-(7′-carboxy-heptyl)-1,4-naphthoquinone] added to urine samples just before analysis. Using this methodology, we confirmed that the 5C- and 7C-aglycones were major catabolites of both phylloquinone (vitamin K_1) and menaquinones (vitamin K_2) in humans. We propose that the measurement of urinary vitamin K metabolite excretion is a candidate noninvasive marker of total vitamin K status.**—Harrington, D. J., R. Soper, C. Edwards, G. F. Savidge, S. J. Hodges, and M. J. Shearer. **Determination of the urinary aglycone metabolites of vitamin K by HPLC with redox-mode electrochemical detection.** ***J. Lipid Res.*** **2005.** 46: **1053–1060.**

Supplementary key words phylloquinone • menaquinones • urinary vitamin K metabolites • aglycones • high-performance liquid chromatography with electrochemical detection

Compounds with vitamin K activity have a common 2-methyl-1,4-naphthoquinone nucleus and a variable alkyl substituent at the 3 position. The major naturally occurring K vitamins are the plant form phylloquinone (vitamin K_1; abbreviated K_1) and multiple forms of menaquinones (vitamins K_2; abbreviated MK), predominately of bacterial origin. K_1 has a 20 carbon phytyl side chain, whereas MKs have multiple prenyl side chains, their number being indicated by a suffix (i.e., MK-n). In a typical Western diet, K_1 and MK-n account for ~90% and 10% of vitamin K intake, respectively (1). Dietary MK-n mainly comprises MK-4, MK-7, MK-8, and MK-9 (2), although MKs with longer side chains up to MK-13 are present in human liver (3, 4). Menadione (abbreviated K_3) is a synthetic vitamin K homolog that lacks the side chain at the 3 position and that, despite toxicity concerns and restricted biological activity, is still available in some countries as a pharmaceutical vitamin K preparation in the form of menadiol sodium phosphate (5) or similar water-soluble salt. The biological activity of K_3 in vivo depends entirely on its prenylation to MK-4 (6, 7).

At the cellular level, the cofactor role of vitamin K for the posttranslational conversion of specific peptide-bound glutamate to γ-carboxyglutamate (Gla) is well established, as is the intimately associated metabolic cycle whereby the vitamin K 2,3-epoxide metabolite generated during γ-glutamyl carboxylation is salvaged (8, 9).

Other aspects of the intermediary metabolism of vitamin K, including the processes leading to vitamin K catabolism and excretion, are much less understood. During the 1970s, human studies using radiolabeled tracer and unlabeled pharmacological doses showed that K_1 was rap-

Abbreviations: 5C-aglycone, 2-methyl-3-(3′-3′-carboxymethylpropyl)-1,4-naphthoquinone; 7C-aglycone, 2-methyl-3-(5′-carboxy-3′-methyl-2′-pentenyl)-1,4-naphthoquinone; ECD, electrochemical detection; Gla, γ-carboxyglutamate; IS, internal standard; K_1, phylloquinone; K_3, menadione; MK, menaquinone; SPE, solid-phase extraction.

[1] To whom correspondence should be addressed.
e-mail: domonic.harrington@gstt.nhs.uk

Manuscript received 15 November 2004 and in revised form 24 January 2005.

Published, JLR Papers in Press, February 1, 2005.
DOI 10.1194/jlr.D400033-JLR200

idly and extensively catabolized via the urine and bile (10–12). These studies established that the major aglycone metabolites of K_1 were two side chain-shortened carboxylic acids with the structures 2-methyl-3-(3′-3′-carboxymethylpropyl)-1,4-naphthoquinone (5C-aglycone; side chain length of 5 carbon atoms; structure IV in **Fig. 1**) and 2-methyl-3-(5′-carboxy-3′-methyl-2′-pentenyl)-1,4-naphthoquinone (7C-aglycone; side chain length of 7 carbon atoms; structure III in Fig. 1) (10–12). Both 5C- and 7C-aglycones were excreted as water-soluble conjugates, mainly with glucuronic acid.

At the time of isolation and characterization of these two metabolites, the role of vitamin K was thought to be limited to its classical coagulation function, and human deficiency at the population level was only considered a problem during the first 6 months of life (13). However, the subsequent discovery of many more vitamin K-dependent proteins (also called Gla proteins), with a widespread tissue distribution, has led to a reevaluation of the general physiological function and health roles of vitamin K. Putative roles of Gla proteins now extend to a diversity of functions, such as the regulation of bone turnover and calcification (14, 15), inhibition of vascular calcification (16), and roles in vascular repair processes (17), cell cycle regulation, cell-cell adhesion, and signal transduction (18). Of particular note is the accumulating body of evidence that has linked suboptimal vitamin K reserves in bone to an increased risk of osteoporotic fracture (19, 20) or to reduced bone mineral density (21). To address this issue and other questions, there has been a need to develop new biochemical measures to monitor vitamin K status in human populations (22). Current measures include the direct measurement of circulating vitamin K (as an indicator of tissue stores) and functional assessments of the γ-carboxylation status of specific Gla proteins such as prothrombin and osteocalcin, representing hepatic and bone γ-carboxylation capacity, respectively. In addition, measurements of urinary free Gla offer an overall assessment of the γ-carboxylation status of Gla proteins. Each of these status assessments has a number of methodological and interpretational drawbacks (23–25). Although useful in many situations, the measurement of circulating vitamin K to assess tissue stores has the major disadvantage that only K_1 is commonly measured, to the detriment of MKs. MKs from the diet (1, 2) and possibly from intestinal synthesis (3, 4) may make a significant contribution to total daily vitamin K intake (1), and the liver stores of vitamin K are predominately long-chain MKs (3, 4).

Here, we describe the development of chromatographic techniques to quantify the two major urinary aglycones of vitamin K with high sensitivity, enabling their measurement at low physiological concentrations. We chose electrochemical detection (ECD) in the redox mode for the final analytical HPLC stage because this method is especially suitable for quinone compounds and has been used successfully for the determination of vitamin K in serum (26, 27). To determine whether the 5C- and 7C-aglycones are common to all K vitamins, we obtained urine samples from adults before and after supplementation with different doses of K_1, MK-4, MK-7, and K_3. We also analyzed urine samples from newborn infants, who, as a group, are known to have precariously low vitamin K stores and are routinely given vitamin K prophylaxis at birth.

MATERIALS AND METHODS

Reagents

5C-aglycone, 7C-aglycone, and the compound [2-methyl-3-(7′-carboxy-heptyl)-1,4-naphthoquinone] used for the internal standard (IS) are not commercially available and were synthesized for this study (28). The γ-lactone form of the 7C-aglycone [2-methyl-3-(5′-carboxy-3′-hydroxy-3′-methylpentyl)-1,4-naphthoquinone lactone], also known as vitamin K γ-lactone, was a gift to M.J.S. from Hoffmann-La Roche and Co. (Basel, Switzerland). Organic solvents were of HPLC grade (Rathburns Chemicals, Walkerburn, Scotland). Water for ECD was purified using a Purite Neptune water purification system (Jencons-PLS, Leighton Buzzard, UK). Potassium hydroxide (Ultra grade), 1-methyl-3-nitro-1-nitrosoguanidine (MNNG), and ethylenediaminetetraacetic acid (disodium salt, dihydrate, Analar grade) were obtained from Sigma-Aldrich (Dorset, UK). Anhydrous sodium acetate, glacial acetic acid, and 3 M HCl, all AristaR grade, were obtained from BDH (Lutterworth, Leicstershire, UK). Nitrogen gas (oxygen free) for removal of solvents was obtained from BOC (Guildford, Surrey, UK).

Apparatus

Solid-phase extraction (SPE) procedures were performed using Isolute™ SPE C_{18} (100 mg, 1 ml reservoir) cartridges (Jones

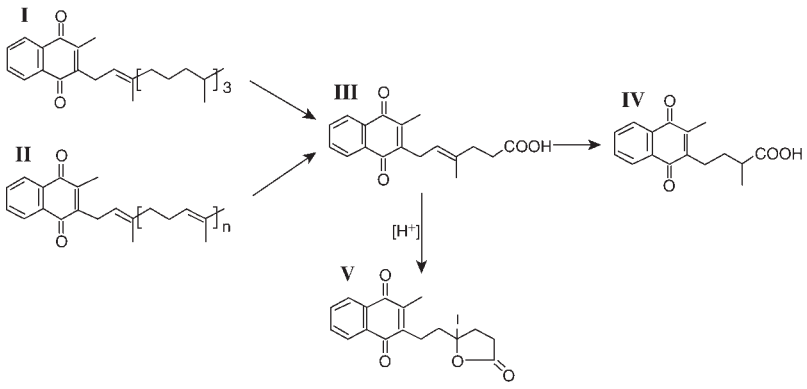

Fig. 1. Structural formulas of phylloquinone (vitamin K_1; abbreviated K_1; I), menaquinones (vitamin K_2; abbreviated MK; II), 7C-aglycone metabolite [2-methyl-3-(5′-carboxy-3′-methyl-2′-pentenyl)-1,4-naphthoquinone; III], 5C-aglycone metabolite [2-methyl-3-(3′-3′-carboxymethylpropyl)-1,4-naphthoquinone; IV], vitamin K γ-lactone (V).

Chromatography, Mid Glamorgan, UK) and Sep-Pak™ silica plus cartridges (Waters Ltd., Elstree, Hertfordshire, UK) with a SPE vacuum manifold.

For HPLC, we used a Gynkotek model 480 pump with pulse damper, a Waters™ 717 plus Autosampler, and an in-line DeJour X-Act HPLC degassing unit. For on-line detection, we used a DECADE electrochemical detector (Antec, Leyden, The Netherlands) equipped with an in-line cell (model 5011) containing dual porous graphite coulometric electrodes in series (ESA Analytical Ltd., Aylesbury, Buckinghamshire, UK) and an amperometric wall jet electrode situated immediately downstream (Antec model VT-03). Chromatographic data were captured using a data chromatography manager (Waters).

Urine collection and human subject protocols

Twenty four hour and spot urine collections were made into plastic containers. Owing to the photosensitive nature of the metabolites, the urine containers were protected from sources of strong light. Urine collections were stored at room temperature for the duration of the collection period, and aliquots were frozen at −70°C until analysis. No significant metabolite loss occurred under these conditions.

We investigated urinary 5C- and 7C-aglycone excretion in healthy adult volunteers. Measurements were made in spot or 24 h urine collections in both unsupplemented and vitamin K-supplemented subjects. For the supplementation studies, the subjects took different forms of vitamin K orally at doses previously used for the treatment or prevention of several pathologies. The vitamin K compounds used were K_1 (2 mg and 50 mg) (29); two homologs representative of the MK series, MK-4 (45 mg) (30) and MK-7 (1 mg) (31); and K_3 (20 mg) (5). The amounts of 5C- and 7C-aglycone excreted in the urine were measured before and after supplementation.

A number of spot urine samples were also collected from newborn infants (two males, one female) before and after intramuscular K_1 prophylaxis with 1 mg of Konakion Neonatal (Roche, Basel, Switzerland).

All adult volunteers and guardians of newborns gave full written informed consent before participation in these investigations.

Assay procedure

A flow chart showing the extraction and analytical procedures is shown in **Fig. 2**.

Extraction and hydrolysis of metabolites

A C_{18} SPE cartridge was prewashed with 1 ml of methanol followed by 1 ml of deionized water. Aliquots of 0.5 ml (unsupplemented subjects) or 0.05 ml (after vitamin K supplementation) of urine were loaded onto the SPE cartridge and allowed to elute to waste under gravity. To remove urinary salts, the SPE cartridge was washed with 1 ml of deionized water at a flow rate of ∼0.5 ml/min, and the eluent was discarded (32). The conjugated vitamin K metabolites were then eluted into a clean tapered polypropylene test tube (12 ml capacity) with 2 ml of a methanolic stock solution containing 0.15 μg of the IS, and the eluent was evaporated to dryness under a stream of N_2 at 50°C.

Conjugated urinary vitamin K metabolites were hydrolyzed overnight at room temperature (in the dark) with 1.1 ml of methanolic HCl [prepared by combining 1 volume of concentrated (35%) HCl with 4 volumes of methanol] (33). The metabolites, now as their aglycones, were extracted into chloroform by the addition of 1.1 ml of chloroform and 1.0 ml of water to the sample tube (34). The upper methanolic-aqueous layer was removed and discarded. To prevent acid-catalyzed lactonization of the 7C-aglycone during subsequent drying procedures, the lower chloroform layer was washed with 10 ml of deionized water to remove any residual mineral acid. After brief shaking and time to allow the two phases to separate, the upper aqueous layer was discarded and the lower chloroform layer was evaporated to dryness under a stream of N_2 at 50°C.

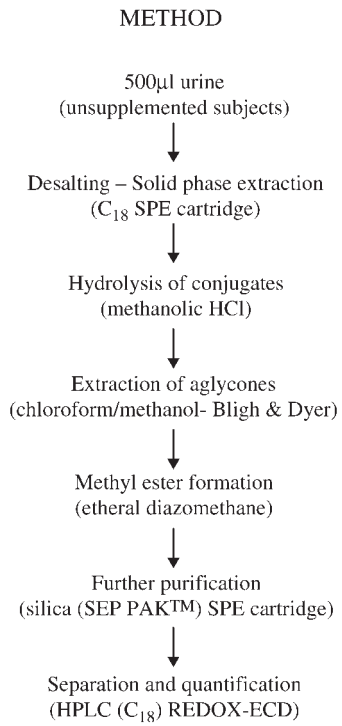

Fig. 2. Flow chart illustrating the analytical stages involved in the determination of the urinary vitamin K aglycone metabolites. ECD, electrochemical detection; SPE, solid-phase extraction.

Methylation of aglycones

The carboxylic acid forms of the vitamin K aglycones were converted to their methyl ester derivatives by reaction with freshly prepared ethereal diazomethane generated on a small scale by a modification of a method described elsewhere (35). In brief, a mixture of 5 M KOH (∼20 ml) and diethyl ether (∼15 ml) was placed into a small, screw-capped bottle (100 ml capacity; Duran), and solid MNNG was gradually added until sufficient diazomethane was generated to turn the upper ether layer yellow (caution: only perform in a well-ventilated fume hood). A volume of 0.5 ml of this ethereal diazomethane was then added to each urine extract. Complete methylation of the carboxylic acid group of the aglycone metabolites was achieved at room temperature within 5 min, after which the diethyl ether was removed under a stream of N_2 at 50°C.

Normal-phase SPE of methylated aglycones

Further fractionation of the urine extract was achieved by normal phase-SPE using Sep-Pak™ cartridges. Each cartridge was attached to a 10 ml glass syringe with a Luer end fitting and activated by drawing through 2 ml of *n*-hexane. Each extract was dissolved in 2 ml of *n*-hexane and then pipetted into the glass syringe attached to the activated cartridge. The *n*-hexane was drawn through the cartridge and the eluent was discarded. Another volume of 8 ml of *n*-hexane was added to the sample tube and the washings were drawn through the cartridge and discarded as before. The vitamin K metabolites and IS were then eluted from the cartridge with 10 ml of *n*-hexane-diethyl ether

(85:15, v/v) and collected into a fresh stoppered, tapered test tube of polypropylene. The solvent was removed under a stream of nitrogen at 50°C.

Reversed-phase HPLC-ECD

The final separation of the methylated aglycones was achieved by reversed-phase HPLC using a Thermo Hypersil-Keystone Hypurity C_{18} column (3 μm particle size, dimensions 100 × 4.4 mm; Hichrom, Reading, Berkshire, UK) and a mobile phase consisting of 60:40 (v/v) methanol-0.05 M sodium acetate buffer (pH 3.0; containing 0.1% EDTA) (26). The flow rate was 1.0 ml/min. Effluent from the column passed through the coulometric dual-electrode cell in which the twin, porous graphite electrodes were set at a negative potential (−1.2 V), thereby reducing the urinary vitamin K quinone metabolites and IS to their quinol states. The effluent then passed to the amperometric wall jet electrode set at +0.3 V, which resulted in the reoxidation of the quinol forms of metabolites and IS to their respective quinones. Chromatograms were generated by monitoring the current (nanoamperes) at the wall jet electrode.

Quantification of 5C- and 7C-aglycone metabolites

From methanolic stock solutions of the 5C- and 7C-aglycones and the IS (methyl ester forms), we prepared a series of standards containing all three analytes with spectrophotometrically determined (EM mM = 18.9 at 248 nm) weight ratios of 5C-aglycone/IS and 7C-aglycone/IS ranging from 0.003 to 0.121. Each chromatographic run included the direct injection of 10 μl of each calibration standard. For routine analyses (i.e., for physiological urinary concentrations of metabolites), the SPE fractionated extracts were reconstituted in 40 μl of methanol and a volume of 10 μl was injected onto the reversed-phase HPLC system. For the determination of the much higher levels after vitamin K supplementation, the extract was reconstituted in 100 μl of methanol and 10 μl was injected. Metabolite concentrations were quantified by the method of peak height ratios. For each chromatogram generated, the peak heights of 5C- and 7C-aglycones were expressed as a ratio to the IS peak. A calibration plot of peak height ratios of the standards versus their weight ratios gave excellent linearity (typically, $r^2 \geq 0.999$) and was used to calculate the equivalent weight ratios of aglycone/IS for each unknown. Multiplication of this weight ratio by the amount of IS originally added gave the amounts of aglycones in the volume of urine processed.

RESULTS

Optimization of extraction and conjugate hydrolysis procedures

The initial SPE procedure using C_{18} cartridges was an effective strategy for desalting the urine and concentrating the urinary metabolites in their conjugated forms. True recovery experiments at this C_{18} SPE stage were not possible because there is limited information on the chemical nature and variety of conjugated metabolites in vivo and appropriate standards are unavailable. Nevertheless, recovery experiments using the synthetic 5C-aglycone, 7C-aglycone, and IS showed that there was no significant loss of vitamin K moieties at this stage. Recoveries were reduced if lower eluting volumes (<2 ml) of methanol were used or if the urine was acidified (with HCl) to promote protonization of the carboxylic acid metabolites. Trials with other SPE cartridge chemistries with side chain lengths < C_{18} also resulted in reduced recovery. Analyte recovery was found to vary inversely with the flow rates of both the first aqueous wash and the second methanolic elution steps. Omission of this initial SPE stage increased the baseline current in the final electrochemical reversed-phase HPLC analytical stage, resulting in a decrease in assay sensitivity.

After the initial SPE stage, conjugated metabolites of vitamin K were hydrolyzed with methanolic HCl. At room temperature, hydrolysis of conjugated metabolites with methanolic HCl was complete within 16 h. After hydrolysis, the lipid-soluble aglycones and IS were efficiently extracted into chloroform after the addition of greater volumes of chloroform and water to form a two-phase system, as described for the final stage of the Bligh and Dyer (34) total lipid extraction technique. A second extraction of the methanolic digest with chloroform did not improve the recovery of aglycones.

Methyl ester derivatization of carboxylic acid aglycones

There were two main reasons for adopting a pre-HPLC methylation procedure to quantitatively convert the aglycone carboxylic acids to their respective methyl esters. First, exposure to methanol during the chromatographic procedures resulted in some inadvertent methylation, which was enhanced by the use of methanolic HCl to deconjugate the aglycones. Second, the reduced polarity of these methylated derivatives improved their retention in our reversed-phase HPLC separation system.

SPE purification of methylated aglycones

An additional SPE purification stage using silica cartridges was introduced to remove interfering compounds, which prevented baseline resolution of the 5C- and 7C-aglycones in the final HPLC-ECD analytical stage. Optimization of the composition of the eluting solvent was carried out using a standard mixture containing known concentrations of the 5C-aglycone, 7C-aglycone, and IS. A diethyl ether concentration of 15% diethyl ether in *n*-hexane (v/v) was found to give quantitative recoveries of the methylated aglycones and the IS while producing good baseline resolution on HPLC-ECD. Recovery of the vitamin K aglycones and IS after this and previous purification stages was consistently >90% for each analyte, with no interanalyte bias.

Optimization of HPLC-ECD conditions

Hydrodynamic voltammograms of the 5C- and 7C-aglycone methyl esters together with the IS are shown in **Fig. 3**. A voltage of −1.2 V was selected as the applied potential in the upstream flow-through coulometric twin electrodes for quantitative reduction of the quinone forms of metabolites and IS to their corresponding quinols (this was the maximum possible voltage to the electrodes of the ESA coulometric cell when controlled by the DECADE instrument). In selecting +0.3 V as the applied potential for the downstream amperometric electrode (for the reoxidation of the respective quinols), a minimal degree of assay sensitivity was sacrificed. However, this reduced interfer-

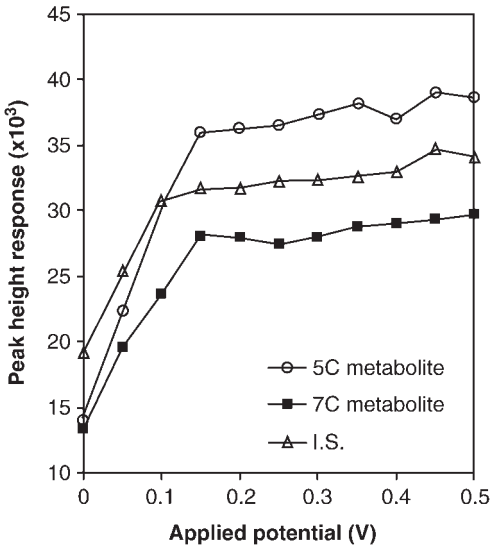

Fig. 3. Hydrodynamic voltammograms for the methylated urinary vitamin K 5C- (open circles) and 7C- (closed squares) aglycone metabolites and the IS (open triangles). The amounts of each compound injected were 16.4 fmol (4.7 pg), 15.7 fmol (4.9 pg), and 956.1 fmol (314 pg), respectively.

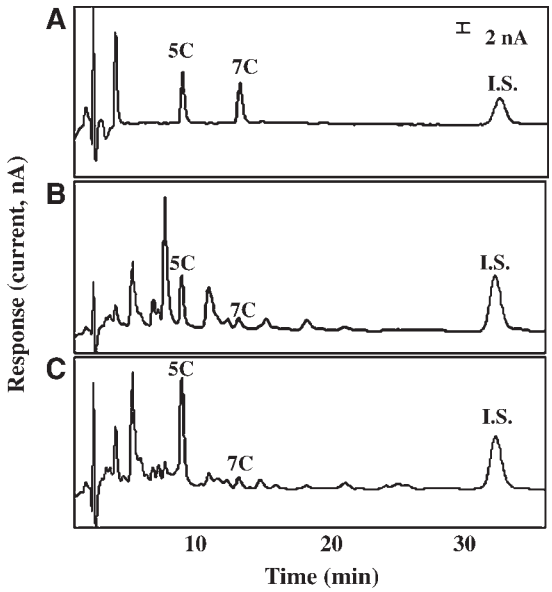

Fig. 4. Representative chromatograms obtained for calibration standards and urinary extracts. A: Chromatogram of a calibration standard showing the resolution of the methylated synthetic 5C-aglycone, 7C-aglycone, and the IS. On-column injection amounts were 166 fmol (47.5 pg) for the 5C-aglycone, 157 fmol (49.0 pg) for the 7C-agylcone, and 960 fmol (314 pg) for the IS. B: Chromatogram showing the measurement of aglycone metabolites at physiological urinary concentrations (i.e., from an unsupplemented subject). A volume of 0.5 ml of urine was extracted, and a fraction of 10/40 μl of the purified extract was injected. The measured 5C- and 7C-aglycone concentrations were 14.76 nmol/l (4.22 μg/l) and 2.82 nmol/l (0.88 μg/l), respectively. C: Chromatogram showing the measurement of urinary aglycone metabolites at increased urinary concentrations [i.e., after oral vitamin K supplementation with 50 mg of K_1 (Konakion)]. A volume of 0.05 ml of urine was extracted, and a fraction of 10/100 μl of the purified extract was injected. The measured 5C- and 7C-aglycone concentrations were 336.36 nmol/l (96.20 μg/l) and 29.32 nmol/l (9.16 μg/l), respectively.

ences from coextracted and cochromatographed substances and resulted in reduced background current.

The electrochemical reduction of the quinone to the quinol moiety was temperature-dependent. Increasing the temperature at which the reductive electrochemical cell was housed improved the overall sensitivity of the assay, and we routinely operated the electrochemical cells in the temperature range of 37–42°C. The methanol component of the mobile phase and system temperature were adjusted until the retention times of the 5C-aglycone, 7C-aglycone, and IS at a flow rate of 1 ml/min were ~8, 12, and 32 min, respectively. Both reductive and oxidative electrochemical cells were found to be robust and foul free after prolonged use.

Calibration, sensitivity, and precision of the HPLC-ECD assay

The injection of varying amounts of the 5C- and 7C-aglycones and the IS gave good peak shapes and a linear detector response within and beyond the mass range used for the quantification of the aglycones. Typical chromatograms of a calibration standard solution and urinary extracts from both unsupplemented and vitamin K-supplemented subjects are shown in **Fig. 4**. The on-column lower limit of detection of 5C- and 7C-aglycones (defined as 5× baseline noise) was <3.5 fmol (<1 pg). The intra-assay and interassay coefficients of variation for 5C- and 7C-aglycone determination in urine from a healthy, nonsupplemented volunteer are shown in **Table 1**.

Mineral acid-catalyzed lactonization of the 7C-aglycone side chain

The 7C-aglycone in its acid form differs from the 5C-aglycone in having a double bond at the γ-δ position relative to the carboxyl group of the side chain. Organic acids with this γ,δ-unsaturated structure are unstable to mineral acids and undergo cyclization to a five member γ-lactone ring structure. As has been demonstrated in radioisotopic studies, the 7C-aglycone in its carboxylic acid form is readily

TABLE 1. Assay reproducibility for the determination of the 5C- and 7C-aglycone metabolites of vitamin K in urine

Sample and Measurement	5C-Aglycone	7C-Aglycone
	nmol/l	
Urine sample A		
Interassay reproducibility[a] (n = 45)		
Mean ± SEM	14.13 ± 0.21	3.62 ± 0.06
Coefficient of variation (%)	9.84	12.75
Urine sample B		
Intra-assay reproducibility (n = 10)		
Mean ± SEM	26.43 ± 0.49	5.86 ± 0.06
Coefficient of variation (%)	5.95	2.85

Two separate urine collections from healthy subjects were analyzed for the 5C- and 7C-aglycone metabolites of vitamin K to assess interassay and intra-assay reproducibility.

[a] Interassay reproducibility was assessed over a period of 5 months.

converted to vitamin K γ-lactone (structure V in Fig. 1) under aqueous acidic conditions, such as when mineral acid is used to release the aglycones from their conjugated forms in urine (10–12). To determine whether we could demonstrate γ-lactonization by HPLC, we subjected 0.5 ml aliquots of urine (urine collected from a volunteer who had ingested 50 mg of K_1) to hydrolysis with 1 ml of aqueous 3 M HCl at 70°C for 60 min. After aqueous HCl hydrolysis, the aglycones were extracted with chloroform and subjected to the usual assay procedure. Treatment with mineral acid completely abolished the peak that cochromatographed with the 7C-aglycone standard and resulted instead in an extra peak that cochromatographed with authentic vitamin K γ-lactone (data not shown). A peak of vitamin K γ-lactone was not found in urine samples after hydrolysis of the metabolite conjugates with methanolic HCl or enzyme preparations of β-glucuronidase (also containing sulfatase).

Confirmation of chromatographic peak identification

Identification of the 5C- and 7C-methyl ester aglycone chromatographic peaks isolated from urine was initially achieved by confirming that they had chromatographic and chemical properties identical to those of the authentic synthetic standards. The chemical similarities between analytes and standards included their hydrodynamic voltammograms, their sensitivity to light, and, for the 7C-aglycone, the lability toward mineral acid and conversion to a compound chromatographically indistinguishable from vitamin K γ-lactone. Definitive confirmation of correct peak assignment was provided by in vivo studies that confirmed that the chromatographic measurements responded to supplementation with different forms of vitamin K.

Typical concentrations (corrected for creatinine) of 5C- and 7C-aglycones in urine samples collected on 2 consecutive days from five healthy, fasting adults (subjects A to E) are shown in **Table 2**. Table 2 also shows the increase in urinary concentrations of both aglycones in response to supplementation with K_1, MK-4, MK-7, and K_3 in a dose-dependent manner. These studies provided confirmation of the chromatographic peak identity for the respective aglycones.

DISCUSSION

To our knowledge, this is the first method to have been developed for the routine measurement of the two major

TABLE 2. Urinary concentrations of vitamin K metabolites (5C- and 7C-aglycones) in unsupplemented and vitamin K-supplemented healthy adults and neonates

Subject	Day 1, 5C	Day 1, 7C	Day 2, 5C	Day 2, 7C
	nmol/mmol creatinine		*nmol/mmol creatinine*	
Unsupplemented[a]				
A	2.5	1.1	1.3	0.7
B	1.7	0.5	1.3	0.5
C	0.3	0.1	0.8	0.3
D	0.8	0.2	1.0	0.2
E	0.6	0.1	0.5	<0.1

Subject	Supplement	Dose	Predose, 5C	Predose, 7C	Postdose Sampling Time	Postdose, 5C	Postdose, 7C
		mg	*nmol/mmol creatinine*		*h*	*nmol/mmol creatinine*	
Supplemented[b]							
F	K_1	2	2.5	0.4	8	11.0	0.6
G	K_1	50	1.7	0.4	0–24	161.7	11.6
H	MK-4	45	1.2	0.2	0–24	118.6	14.5
I	MK-4	45	1.7	0.3	0–24	57.2	15.7
J	MK-4	45	1.1	0.1	0–24	76.6	21.4
K	MK-7	1	0.9	0.1	24	4.2	0.6
L	MK-7	1	1.6	0.4	4	4.8	1.4
M	K_3	20	1.3	0.4	5	5.6	1.8
N	K_3	20	2.5	0.5	6	17.8	5.1
Neonate A	K_1 (IM)	1	0.2	0.2	5	5.4	14.0
Neonate B	K_1 (IM)	1	0.6	0.4	24	12.7	4.4
Neonate C	K_1 (IM)	1	0.2	0.2	48	153.8	32.1

K_1, phylloquinone; K_3, menadione; MK, menaquinone.

[a] Unsupplemented healthy adult subjects A to E collected spot urine at 9:00 AM on consecutive days after a 14 h fast.

[b] Supplemented healthy adult subjects F to N took the indicated vitamin K supplement orally and collected urine before and after supplementation. The presupplementation urine samples for subjects F to L were 24 h collections, and those for subjects M and N were spot urine samples (early morning) collected immediately before taking the vitamin K supplement. The postsupplementation urine samples were either spot samples collected at the times indicated (subjects F and K to N) or entire 24 h collections (subjects G to J). The neonates received 1 mg of K_1 intramuscularly (IM) on the first day of life. The presupplementation neonatal samples represented the first void spot urine samples after delivery. The postsupplementation neonatal samples were spot urine samples collected at the indicated times after intramuscular K_1.

urinary aglycones of vitamin K. The method is sensitive, requiring small sample volumes, and the interassay precision was ~10% for both 5C- and 7C-aglycones. Although we used the same principle of redox mode electrochemical detection as used for a previous assay of the parent K vitamins (27, 36), the metabolite assay has proved to be much less susceptible to periodic loss of sensitivity. In the serum vitamin K assay, this often rapid loss of sensitivity is thought to be attributable to passivation of the electrodes by adsorbed species of vitamin K and coextracted nonpolar lipids (27, 36). The lack of significant electrode passivation in the present assay may be related to the greater polarity of the metabolites and coextracted urinary components, which makes them less likely to adsorb to the carbon electrodes than are the highly lipophilic vitamin K and other lipids present in serum extracts.

The 5C- and 7C-aglycones measured in this assay were derived by the chemical hydrolysis of conjugates by methanolic HCl. This method of hydrolysis was previously used for the isolation of the urinary aglycones of ubiquinone-7 (33) and K_1 (37) from rabbit urine. Previous work from our laboratory has suggested that the majority of the 5C- and 7C-aglycones in human urine are excreted as glucuronides (10, 11). We also confirmed in this study (data not shown) that incubation of urine with β-glucuronidase released the same two 5C- and 7C-aglycones and gave chromatograms identical to those after methanolic HCl hydrolysis. This does not exclude the possibility of other conjugates being present, because K_3, which lacks the side chain, has been shown to be excreted as both glucuronide and the sulfate conjugates of the quinol form in rats (38, 39) and rabbits (40). For routine quantitative analyses, we chose methanolic HCl over enzymic hydrolysis for reasons of its greater convenience and efficiency and to ensure the complete hydrolysis of all conjugates present.

The results in Table 2 show that in both unsupplemented and supplemented adults, the excretion of the 5C-aglycone is greater than the excretion of the 7C-aglycone. This is in agreement with early radioisotopic measurements of these two aglycones after the administration of tritium-labeled K_1 to adults (10, 11). In the latter studies, the intravenous administration of single doses of 45–1,000 μg of labeled K_1 resulted in a gradually increasing proportion of the 7C-aglycone (assayed as vitamin K γ-lactone after acid hydrolysis) from an initial 13% to some 30–40% in successive urine collections made over the first 24 h (10). In rabbits, the administration of a huge (91 mg) intravenous dose of K_1 changed the percentage proportion of 7C-aglycone (in 24 h urine collections) from 13% in control animals to 57% after supplementation (37). These data suggest that whereas physiological amounts of K_1 are largely metabolized to the terminal 5C-aglycone, vitamin K supplementation may lead to a greater urinary excretion of the less extensively metabolized 7C-aglycone. Interestingly, one of the infants studied here (Infant A in Table 2) excreted predominantly the 7C-aglycone after supplementation with 1 mg of K_1. Further support that large doses may overload the pathway comes from the previous isolation and definitive identification of a 10C side chain length aglycone after a very large K_1 dose (12). No evidence for this 10C-aglycone was seen on our chromatograms. Although the preliminary supplementation studies shown in Table 2 using the present assay showed dose-related increases in the urinary concentrations of both 5C- and 7C-aglycones, there was no consistent pattern in their relative concentrations. The supplementation studies with K_1 and members of the MK series (MK-4 and MK-7) clearly indicate that the 5C- and 7C-aglycones are common to the major naturally occurring forms of vitamin K (Table 2). Although the excretion of different forms of vitamin K as these two common catabolites has not been formally demonstrated before, it was to be expected in view of previous work that has shown that the isoprenoid side chains of ubiquinone-7 (33), K_1 (11, 37), α-tocopherol (37, 41), and δ-tocopherol (42) are all shortened in the same way and excreted as equivalent metabolites.

The proposed degradation pathway (Fig. 1) of vitamin K by successive ω- and β-oxidation is supported by the previous unambiguous identification of 5C- and 7C-aglycones (11), by the finding of the expected 10C side chain intermediate (12), and by analogy to the findings of others that the same degradation pathway operates for similar isoprenoid compounds (33, 41, 42).

The increase in 5C- and 7C-aglycone excretion in response to supplementation with K_3 is noteworthy. The generation of MK-4 from K_3 through the addition of a geranyl-geranyl side chain is well described (6, 7) and is the probable source of the increased excretion of the 5C- and 7C-aglycones after K_3 supplementation. It may be possible to use this metabolite assay as an index of the tissue conversion of K_3 to MK-4.

Our ongoing work is directed at evaluating the present assay as a new marker of vitamin K status. An obvious advantage over current serum measurements of vitamin K is that urinary excretion reflects the degradation of all K vitamins, whereas most serum assays are only directed at the assay of a single vitamer, K_1. Thus, the measurement of urinary excretion might be suitable as a measure of overall vitamin K status. The relative contribution to total urinary excretion from K_1 and MKs remains to be determined. It seems likely that the majority of daily variability stems from excretion of K_1, because this is by far the major dietary form, and long-chain MKs are thought to have a slower turnover (43). In addition to the prerequisite for normal renal function, a possible disadvantage of urinary measurements as a marker of vitamin K stores is that metabolites of vitamin K are also excreted via the bile, so that urinary excretion will not reflect the total elimination of vitamin K from the body.

The authors thank Dr. Paul Clarke and the staff of the Neonatal Unit at Hope Hospital (Salford, UK) for their perseverance and diligence in providing us with urine collected from neonates. The authors also thank the adult volunteers who participated in the vitamin K supplement studies. Special thanks are due to Dr. Leon Schurgers (Maastricht University, Maastricht, The Netherlands), who provided us with urine samples before and after supplementation with MK-7. The authors are also

grateful to David Card for his assistance in the preparation of the manuscript.

REFERENCES

1. Schurgers, L. J., J. M. Geleijnse, D. E. Grobbee, H. A. P. Pols, A. Hofman, J. C. M. Witteman, and C. Vermeer. 1999. Nutritional intake of vitamin K-1 (phylloquinone) and K-2 (menaquinone) in The Netherlands. *J. Nutr. Environ. Med.* **9:** 115–122.
2. Schurgers, L. J., and C. Vermeer. 2000. Determination of phylloquinone and menaquinones in food. Effect of food matrix on circulating vitamin K concentrations. *Haemostasis.* **30:** 298–307.
3. Shearer, M. J. 1992. Vitamin K metabolism and nutriture. *Blood Rev.* **6:** 92–104.
4. Suttie, J. W. 1995. The importance of menaquinones in human nutrition. *Annu. Rev. Nutr.* **15:** 399–417.
5. British National Formulary. 2004. British Medical Association and Royal Pharmaceutical Society of Great Britain. No. 48 (September).
6. Martius, C., and H. O. Esser. 1958. Über die Konstitution des im Tierkörper aus Methylnaphthochinon gebildeten K-Vitamines. *Biochem. Z.* **331:** 1–9.
7. Taggart, W. V., and J. T. Matschiner. 1969. Metabolism of menadione-6,7-^{3}H in the rat. *Biochemistry.* **8:** 1141–1146.
8. Olson, R. E. 1999. Vitamin K. *In* Modern Nutrition in Health & Disease. 9th ed. M. E. Shils, J. A. Olson, M. Shike, and A. C. Ross, editors. Williams & Wilkins, Baltimore, MD. 363–380.
9. Suttie, J. W. 1992. Vitamin K and human nutrition. *J. Am. Diet. Assoc.* **92:** 585–590.
10. Shearer, M. J., and P. Barkhan. 1973. Studies on the metabolites of phylloquinone (vitamin K_1) in the urine of man. *Biochim. Biophys. Acta.* **297:** 300–312.
11. Shearer, M. J., A. McBurney, and P. Barkhan. 1974. Studies on the absorption and metabolism of phylloquinone (vitamin K_1) in man. *Vitam. Horm.* **32:** 513–542.
12. McBurney, A., M. J. Shearer, and P. Barkhan. 1980. Preparative isolation and characterization of the urinary aglycones of vitamin K_1 (phylloquinone) in man. *Biochem. Med.* **24:** 250–267.
13. Shearer, M. J. 1995. Vitamin K. *Lancet.* **345:** 229–234.
14. Vermeer, C., M. H. Knapen, and L. J. Schurgers. 1998. Vitamin K and metabolic bone disease. *J. Clin. Pathol.* **51:** 424–426.
15. Shearer, M. J. 2000. Role of vitamin K and Gla proteins in the pathophysiology of osteoporosis and vascular calcification. *Curr. Opin. Clin. Nutr. Metab. Care.* **3:** 433–438.
16. Shanahan, C. M., D. Proudfoot, A. Farzaneh-Far, and P. L. Weissberg. 1998. The role of Gla proteins in vascular calcification. *Crit. Rev. Eukaryot. Gene Expr.* **8:** 357–375.
17. Benzakour, O., and C. Kanthou. 2000. The anticoagulant factor, protein S, is produced by cultured human vascular smooth muscle cells and its expression is up-regulated by thrombin. *Blood.* **95:** 2008–2014.
18. Tsaioun, K. I. 1999. Vitamin K-dependent proteins in the developing and aging nervous system. *Nutr. Rev.* **57:** 231–240.
19. Szulc, P., M. C. Chapuy, P. J. Meunier, and P. D. Delmas. 1993. Serum undercarboxylated osteocalcin is a marker of the risk of hip fracture in elderly women. *J. Clin. Invest.* **91:** 1769–1774.
20. Luukinen, H., S. M. Käkönen, K. Pettersson, K. Koski, P. Laippala, T. Lövgren, S. L. Kivelä, and H. K. Väänänen. 2000. Strong prediction of fractures among older adults by the ratio of carboxylated to total serum osteocalcin. *J. Bone Miner. Res.* **15:** 2473–2478.
21. Szulc, P., M. Arlot, M. C. Chapuy, F. Duboeuf, P. J. Meunier, and P. D. Delmas. 1994. Serum undercarboxylated osteocalcin correlates with hip bone mineral density in elderly women. *J. Bone Miner. Res.* **9:** 1591–1595.
22. Sokoll, L. J., and J. A. Sadowski. 1996. Comparison of biochemical indexes for assessing vitamin K nutritional status in a healthy adult population. *Am. J. Clin. Nutr.* **63:** 566–573.
23. Cham, B. E., J. L. Smith, and D. M. Colquhoun. 1999. Interdependence of serum concentrations of vitamin K_1, vitamin E, lipids, apolipoprotein A_1, and apolipoprotein B: importance in assessing vitamin status. *Clin. Chim. Acta.* **287:** 45–57.
24. McKeown, N. M., P. F. Jacques, C. M. Gundberg, J. W. Peterson, K. L. Tucker, D. P. Kiel, P. W. Wilson, and S. L. Booth. 2002. Dietary and nondietary determinants of vitamin K biochemical measures in men and women. *J. Nutr.* **132:** 1329–1334.
25. Gundberg, C. M., S. D. Nieman, S. Abrams, and H. Rosen. 1998. Vitamin K status and bone health: an analysis of methods for determination of undercarboxylated osteocalcin. *J. Clin. Endocrinol. Metab.* **83:** 3258–3266.
26. Hart, J. P., M. J. Shearer, P. T. McCarthy, and S. Rahim. 1984. Voltammetric behaviour of phylloquinone (vitamin K_1) at a glassy-carbon electrode and determination of the vitamin in plasma using high-performance liquid chromatography with electrochemical detection. *Analyst.* **109:** 477–481.
27. Hart, J. P., M. J. Shearer, and P. T. McCarthy. 1985. Enhanced sensitivity for the determination of endogenous phylloquinone (vitamin K_1) in plasma using high-performance liquid chromatography with dual-electrode electrochemical detection. *Analyst.* **110:** 1181–1184.
28. Soper, R. J. 2004. The Synthesis and Biological Activities of Natural Quinone Metabolites. PhD Dissertation. University of Essex, Essex, UK.
29. McCarthy, P. T., A. D. Cox, D. J. Harrington, R. S. Evely, E. Hampton, A. I. al-Sabah, E. Massey, H. Jackson, and T. Ferguson. 1997. Covert poisoning with difenacoum: clinical and toxicological observations. *Hum. Exp. Toxicol.* **16:** 166–170.
30. Shiraki, M., Y. Shiraki, C. Aoki, and M. Miura. 2000. Vitamin K2 (menatetrenone) effectively prevents fractures and sustains lumbar bone mineral density in osteoporosis. *J. Bone Miner. Res.* **15:** 515–521.
31. Tsukamoto, Y., H. Ichise, H. Kakuda, and M. Yamaguchi. 2001. Intake of fermented soybean (natto) increases circulating vitamin K_2 (menaquinone-7) and γ-carboxylated osteocalcin concentration in normal individuals. *J. Bone Miner. Metab.* **18**: 216–222.
32. Pope, S. A. S., P. T. Clayton, and D. P. R. Muller. 2000. A new method for the analysis of urinary vitamin E metabolites and the tentative identification of a novel group of compounds. *Arch. Biochem. Biophys.* **381:** 8–15.
33. Imada, I., M. Watanabe, N. Matsumoto, and H. Morimoto. 1970. Metabolism of ubiquinone-7. *Biochemistry.* **9:** 2870–2878.
34. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Med. Sci.* **37:** 911–917.
35. Fales, H. M., T. M. Jaouni, and J. F. Babashak. 1973. Simple device for preparing etheral diazomethane without resorting to codistillation. *Anal. Chem.* **45:** 2302–2303.
36. McCarthy, P. T., D. J. Harrington, and M. J. Shearer. 1997. Assay of phylloquinone in plasma by high-performance liquid chromatography with electrochemical detection. *Methods Enzymol.* **282:** 421–433.
37. Watanabe, M., M. Toyoda, I. Imada, and H. Morimoto. 1974. Ubiquinone and related compounds. XXVI. The urinary metabolites of phylloquinone and α-tocopherol. *Chem. Pharm. Bull.* **22:** 176–182.
38. Losito, R., C. A. Owen, Jr., and E. V. Flock. 1967. Metabolism of [^{14}C]menadione. *Biochemistry.* **6:** 62–68.
39. Losito, R., C. A. Owen, Jr., and E. V. Flock. 1968. Metabolic studies of vitamin K1-14C and menadione-14C in the normal and hepatectomized rats. *Thromb. Diath. Haemorrh.* **19:** 383–388.
40. Richert, D. A. 1951. Studies on the detoxification of 2-methyl-1,4-naphthoquinone in rabbits. *J. Biol. Chem.* **189:** 763–768.
41. Schuelke, M., A. Elsner, B. Finckh, A. Kohlschütter, C. Hübner, and R. Brigelius-Flohé. 2000. Urinary α-tocopherol metabolites in α-tocopherol transfer protein-deficient patients. *J. Lipid Res.* **41:** 1543–1551.
42. Chiku, S., K. Hamamura, and T. Nakamura. 1984. Novel urinary metabolites of D-δ-tocopherol in rats. *J. Lipid Res.* **25:** 40–48.
43. Shearer, M. J., A. Bach, and M. Kohlmeier. 1996. Chemistry, nutritional sources, tissue distribution and metabolism of vitamin K with special reference to bone health. *J. Nutr.* **126 (Suppl.):** 1181–1186.