Excretion of the Urinary 5C- and 7C-Aglycone Metabolites of Vitamin K by Young Adults Responds to Changes in Dietary Phylloquinone and Dihydrophylloquinone Intakes1,2

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Abstract

The physiological function and putative health roles of vitamin K-dependent proteins now extend beyond their classical role in hemostasis and include bone mineralization, arterial calcification, apoptosis, phagocytosis, growth control, chemotaxis, and signal transduction. Current assessments of vitamin K status do not reflect the variety of molecular forms of vitamin K. We assessed whether urinary excretion of 2-methyl-3-(5'-carboxy-3'-methyl-2'-pentenyl)-1,4-naphthoquinone (7C-aglycone) and 2-methyl-3-(3'-3'-carboxymethylpropyl)-1,4-naphthoquinone (5C-aglycone), vitamin K metabolites common to both phylloquinone and the menaquinone series, reflect dietary vitamin K intake. In a randomized crossover study, 9 adults resided in a metabolic unit for two 30-d periods separated by a free-living period of ≈4 wk. During each residency, subjects consumed 3 sequential diets: a control diet ($93 \mu$g phylloquinone/d) for 5 d, a phylloquinone-restricted diet ($11 \mu$g/d) for 15 d, followed by a randomly assigned repletion diet for 10 d with either phylloquinone ($206 \mu$g/d) or dihydrophylloquinone ($240 \mu$g/d). During the second residency, the alternative repletion diet was assigned. Urinary excretion of the 5C- and 7C-aglycones was measured in sequential 24-h collections. The 5C-aglycone accounted for ~75% of total excretion and declined in response to phylloquinone restriction (P = 0.001) to ~30% of that during the control diet period. Repletion with phylloquinone and dihydrophylloquinone doubled the excretion rate of the major 5C-aglycone by 24 h (P < 0.001), and tripled excretion by 4 d. There was a linear relationship between the logarithm of total urinary excretion and dietary vitamin K intake (r = 0.699, P < 0.001). We conclude that the urinary excretion of vitamin K metabolites reflects dietary phylloquinone intake and offers the first candidate marker of global vitamin K status.


Introduction

Compounds with vitamin K activity share a common 2-methyl-1, 4-naphthoquinone nucleus but have a variable alkyl substituent at the 3 position. Phylloquinone (vitamin K1), the primary dietary form of vitamin K, is synthesized by plants and has a phytol side chain (Fig. 1, I). The menaquinones (vitamin K2) are of bacterial origin and have multi-prenyl side chains; their number is indicated by a suffix (i.e., menaquinone-n), (Fig. 1, II).

Vitamin K is an essential cofactor for the posttranslational modification of specific peptide-bound glutamate residues to γ-carboxyglutamate within vitamin K-dependent proteins (I).

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The physiological function and putative health roles of vitamin K-dependent proteins include hemostasis, bone mineralization, arterial calcification, apoptosis, phagocytosis, growth control, chemotaxis, and signal transduction (1). In the UK, the daily dietary reference value for vitamin K is 1 $\mu$g/kg, an intake regarded as adequate with respect to the coagulation function of vitamin K, but which does not consider the functionality of nonhepatic vitamin K-dependent proteins (2). The U.S. has set an Adequate Intake, based on representative dietary intake data from healthy individuals, of 120 and 90 $\mu$g/d for men and women, respectively (3).

The catabolic pathway that results in urinary excretion of vitamin K metabolites is exclusively hepatic (4) and is likely to involve oxygen attack at the ω-carbon, followed by a side chain shortening of the resultant carboxylic acid by β-oxidation (5,6). This route of catabolism is analogous to that for other natural isoprenoids, such as ubiquinone, tocopherols, and tocoquinones (7). The resultant aglycone metabolites are then conjugated, predominately with glucuronic acid, to facilitate their biliary and urinary elimination (5,6,8).
Materials and Methods

Subjects. This study was based on the availability of archived 24-h urine specimens from a subset of 9 healthy subjects, aged 27–39 y [5 men, median age (range) 27 (25–39) y; 4 women, median age 33 (28–35) y], who participated in the original study (10). The study was approved by the Tufts-New England Medical Center Institutional Review Board and a written consent form was obtained from each subject.

Experimental protocol. Details of the experimental protocol and the composition of the diets were previously published (10). A brief outline is given here. In a randomized crossover design, each subject resided in the Metabolic Research Unit at the Jean Mayer US Department of Agriculture Human Nutrition Research Center on Aging at Tufts University for two 30-d residency periods. There was a free-living period of ≥4 wk between each residency period, during which time each subject consumed a self-selected diet.

Each residency period consisted of 3 diets: a 5-d control diet, a 15-d depletion diet, and a 10-d repletion diet. The control and depletion diets were identical in each residency period. The repletion diet contained either phylloquinone or dihydrophylloquinone, with the order of repletion being randomized among subjects. All meals were provided on a 2-d rotating plan; were designed to meet the dietary reference intakes for energy, protein, minerals, and vitamins for each subject’s age and sex (3,13), except for phylloquinone, and were prepared under the supervision of a dietician.

The 5-d control diet contained 93.1 μg/d of phylloquinone, which approximates an adequate daily intake for this vitamin (3). There was no detectable dihydrophylloquinone in the control diet (11). The depletion diet provided 11 μg/d of phylloquinone. For the 2 repletion diets, purified sources of phylloquinone (Sigma Chemical) or dihydrophylloquinone (>99% purity) were added to corn oil in muffins, which were generated by saturation of the 2,3 double bond in the phytol side chain of phylloquinone during hydrogenation of phylloquinone-rich vegetable oils (11). Interest in this artifactual form of vitamin K stems from its ubiquitous presence in the US diet, although there is a current shift away from the use of partially hydrogenated fat in commercial products (12).

Results

Urinary 5C-aglycone excretion. On entry to the study, urinary excretion of the 5C-aglycone (data from both residency periods combined) was 4.15 ± 0.53 μg/d. After 5 d of the control diet, 5C-aglycone excretion had not significantly changed at 3.55 ± 0.55 μg/d. After 15 d of phylloquinone restriction, urinary 5C-aglycone excretion fell progressively to 2.89 ± 1.10 μg/d (P = 0.001). After the first 24 h of the repletion phase (d 20–21), excretion had increased dramatically to 6.23 ± 0.77 μg/d (P < 0.001) after phylloquinone and to 6.76 ± 0.73 μg/d (P < 0.001) after dihydrophylloquinone supplementation, respectively (Fig. 2A). Urinary output of the 5C-aglycone further increased before reaching a plateau after ~4 d of supplementation. By the end of the 10 d repletion period, the excretion of the 5C-aglycone had increased to 8.48 ± 0.87 μg/d after phylloquinone and to 12.74 ± 0.87 μg/d after dihydrophylloquinone repletion, both values being higher than those after the first 24 h of repletion (P < 0.001).

The daily urinary excretion of the 5C-aglycone was higher during the dihydrophylloquinone repletion residency period than the phylloquinone residency period (P < 0.001) (Fig. 2A).

Urinary 7C-aglycone excretion. Urinary excretion of the 7C-aglycone (phylloquinone and dihydrophylloquinone residencies combined) was 1.31 ± 0.16 μg/d on entry to the study, 1.33 ± 0.16 μg/d at the end of the 5-d control diet, and 1.10 ± 0.19 μg/d after 15 d of phylloquinone depletion (Fig. 2B). As
observed for the 5C-aglycone, urinary excretion of the 7C-aglycone rose after phylloquinone supplementation, reaching a plateau after 4 d. By the end of the 10-d phylloquinone repletion phase, the daily urinary excretion of the 7C-aglycone had increased from 1.10 ± 0.19 μg at the end of the depletion phase to 2.71 ± 0.26 μg (P = 0.001). Conversely, the 7C-aglycone did not respond to dihydrophylloquinone supplementation (Fig. 2B).

**Relationship of 5C- to 7C-aglycone excretion and differences in excretion patterns between phylloquinone and dihydrophylloquinone.** The relative proportions in which the major 5C-aglycone and minor 7C-aglycone were excreted on entry to the study, at the end of the 5-d control phase, and after 15 d of phylloquinone depletion were very similar with the major 5C-aglycone, accounting (on a molar basis) for 77.5, 74.4, and 74.1% of total metabolite excretion at these time points. This proportion of 5C-aglycone excretion remained unchanged (77.3%) after 10 d supplementation with phylloquinone but was 95.5% after supplementation with dihydrophylloquinone. The pattern of excretion of the 7C-aglycone indicates that, during the dihydrophylloquinone arm of the study, the excretion of the 7C-aglycone continued to fall during both the depletion and repletion phases (Fig. 2B). This suggests that dihydrophylloquinone is not metabolized to the 7C-aglycone. This is also predicted by the lack of the 2', 3' double bond in the side chain of dihydrophylloquinone (Fig. 1, V). The unhindered metabolism of dihydrophylloquinone to the 5C-aglycone suggests the possibility of the existence of an equivalent 7C-aglycone without the 2', 3' double bond (Fig. 1, VI).

**Total urinary vitamin K metabolite excretion as a function of dietary phylloquinone intake.** We calculated the total (5C- + 7C-) aglycone excretion in response to each dietary exposure. Daily metabolite excretion remained constant during the control diet period, whereas equilibration to the depletion and phylloquinone repletion diets was essentially achieved after 4 d. As a best estimate of urinary metabolite output in response to a specific level of phylloquinone intake, and to minimize intra-individual fluctuations, we calculated the mean daily excretion during the last 3 d of each diet period. Daily total excretion was 3.52 ± 0.19 μg (phylloquinone intake 11 μg/d), 5.54 ± 0.21 (93 μg/d), and 10.63 ± 0.32 (206 μg/d). A plot (graph not shown) of these 3 levels of intakes (x axis) vs. the logarithm of total urinary excretion (y axis) was approximately linear (r = 0.699, P < 0.001, equation \( y = 3.254e^{0.0038x} \)). The intercept on the y-axis of 3.25 μg/d (−11 nmol/d if all 5C-aglycone) represents an extrapolated theoretical estimate of total urinary excretion in response to a diet totally devoid of phylloquinone, that had been consumed over a similar period of 10–15 d.

The total daily excretion of vitamin K metabolites as a molar proportion of the daily dietary phylloquinone intake was 9.2% for the control diet (molar phylloquinone intake, 206.7 nmol/d) and 8.0% for the repletion diet (intake 457.3 nmol/d). This proportion rose to 49.4% during the last 3 d of the depletion diet (intake 24.4 nmol/d). If we assume a similar molar percentage of ~8–9% of the daily urinary excretion derived from the depletion diet, it may be calculated that, by the end of the depletion period, some 2.0 nmol/d (0.6 μg/d) of the total daily urinary output of 12 nmol/d (3.5 μg/d) could be attributed to dietary sources. This suggests that after dietary depletion, an amount of ~10 nmol/d (2.9 μg/d), representing ~85% of daily excretion, must have derived from an unknown combination of dietary or intestinally synthesized menaquinones and the turnover of body stores of vitamin K. This value of daily metabolite excretion from non-immediate dietary phylloquinone sources after severe dietary restriction agrees closely with our previously calculated theoretical estimate, obtained by graphical extrapolation, of a basal

![FIGURE 1 Structural formulas of phylloquinone (vitamin K1), menaquinones (vitamin K2), and 2',3'-dihydrophylloquinone, and their predominant urinary aglycone metabolites. (a) Phylloquinone (vitamin K1); (b) menaquinones (vitamin K2); (c) 2-methyl-3-(5'-carboxy-3'-methyl-2'-pentenyl)-1,4-naphthoquinone (7C-aglycone metabolite of I and II); (d) 2-methyl-3-(3'-3'-carboxymethylpropyl)-1,4-naphthoquinone (5C-aglycone metabolite of I, II, and VI); (e) 2',3'-dihydrophylloquinone; (f) 2-methyl-3-(5'-carboxy-3'-methylpentyl)-1,4-naphthoquinone (tentative 7C-aglycone metabolite of VI). (a) Saturation of the 2', 3' double bond in the phytyl side chain of phylloquinone during the hydrogenation of phylloquinone-rich vegetable oils; (b) ω-oxidation and subsequent β-oxidation of the isoprenoid side chain; and (c) further β-oxidation.](https://academic.oup.com/jn/article-abstract/137/7/1763/4664527)
metabolite excretion of ~11 nmol/d (≈3.2 μg/d) for a diet totally devoid in phylloquinone.

Discussion

Our main findings were that relatively short-term manipulations of dietary phylloquinone and dihydrophylloquinone intake resulted in corresponding and significant changes in urinary metabolite excretion. The 5C-aglycone accounted for ~75% of the total daily metabolite excretion, over dietary intakes of phylloquinone that ranged from 11 to 206 μg/d, a range that encompasses habitual phylloquinone intakes of populations of the UK and USA (14–17). The data from the dihydrophylloquinone supplementation phase confirmed that this hydrogenated form was metabolized by the same β-oxidative metabolic pathway as phylloquinone to produce the 5C-aglycone. The lack of excretion of the 7C-aglycone in response to dietary dihydrophylloquinone repletion is consistent with the catabolism of this 2’, 3’-hydrogenated form by the same metabolic pathway and suggests that an equivalent hydrogenated 7C-aglycone may be generated and either excreted or further catabolized to the common 5C-aglycone. Further studies are needed to confirm this.

Previous radioisotopic studies have shown that a sizeable fraction of an injected or orally administered bolus of phylloquinone is rapidly metabolized and lost to the body within a few days via urine and bile (6,14). Somewhat surprisingly, similar proportions of an injected dose of phylloquinone have been found to be excreted for a tracer dose of 0.3 μg (14) as for higher doses of 45 μg and 1000 μg phylloquinone (6). After taking into account the extent of absorption, similar fractions are excreted in the urine and feces after an oral dose as when the vitamin is given intravenously (6). The available data suggest that ~20–30% of a bolus of newly absorbed phylloquinone will be excreted in the urine and 30–40% in the feces. This rapid metabolic turnover of phylloquinone explains the significant rise in total urinary output within 24 h of changing from the depletion to the repletion diet. Urinary excretion also responded within 24 h of changing from the control to the depletion diet. A caveat to our results is that fecal output of metabolites was unknown, and therefore any shift of excretion between the 2 routes during the study could not be assessed. Olson et al. (14) noted that after 3–8 wk of dietary phylloquinone depletion (8 μg/d), the fractional rate of excretion of the injected dose of 0.3 μg phylloquinone in the urine (t1/2 24.8 h) was not significantly different from that (t1/2 20.1 h) when subjects consumed the control diet. Moreover, although the recovery of the injected dose in urine was not affected by dietary depletion (30% control; 38% depletion diet), the proportion of the dose recovered in the feces fell from 32% during the control diet period to 13% for the depletion diet period (14). These data lend support to the view that the phylloquinone restricted diet of our study (11 μg/d) would not have significantly affected that fraction of dietary phylloquinone that is excreted in the urine, which we estimated at 8–9%. This low proportion of ingested phylloquinone excreted as these metabolites was based on the very similar fractions of dietary phylloquinone that were excreted in the urine after feeding the control (93 μg/d) and repletion diets (206 μg/d), respectively. The possibility of a higher bioavailability of phylloquinone from the corn-oil supplemented repletion diet cannot be discounted, although the fact that the proportional excretion was the same for both diets argues against this. If all these assumptions are true, we estimate that after 15 d of dietary phylloquinone restriction, ~85% of the daily excretion of 5C- and 7C-aglycones had derived from vitamin K sources other than dietary phylloquinone. These sources would include the menaquinone series and mobilized tissue stores of phylloquinone. Dietary intakes of menaquinones were not assessed in our study, but in a Dutch epidemiological study, they were estimated by FFQ to be ~20 μg/d (18).

The inadequacy of the depletion diet for optimal vitamin K function was shown by the findings in the original study (10), of significant rises in functional markers of γ-carboxylation, undercarboxylated prothrombin (PIVKA-II), and percent undercarboxylated osteocalcin (%ucOC). This suggests that a proportion of the total daily excretion of urinary aglycones after the depletion phase includes losses of “active” vitamin K stores being utilized for γ-carboxylation in both liver and bone. After the phylloquinone repletion phase, the circulatory concentrations of both PIVKA II and %ucOC returned to those observed at study entry (baseline) (10). Within 24 h of starting phylloquinone supplementation, there were significant rises in both circulatory phylloquinone and in urinary output of vitamin K. However, whereas the rise in plasma phylloquinone was muted (rising only to concentrations similar to those during the control diet period), daily urinary metabolite excretion rose rapidly, to approximately double that observed during the control diet period, in line with the doubling of phylloquinone intake. This suggests that the change in urinary output is a more responsive indicator
of short-term supplementation than the change in plasma levels. The unchanged urinary output from baseline to the end of the control period suggests that the dietary phylloquinone intakes of the subjects at study entry had been similar to that during the control diet period. Thus, these new urinary data suggest that the fall in plasma phylloquinone during the control phase (10) may have been due to factors other than to a decreased dietary intake of phylloquinone. One possibility is that the control diet contained less fat than the subjects’ habitual diets, and this may have lowered serum lipids, which are known to be positively associated with plasma phylloquinone concentrations (19,20).

Despite the rapid turnover of a sizeable body pool of phylloquinone, excretion of the 7C-aglycone continued to decline during both the phylloquinone restriction and dihydrophylloquinone repletion phases. Because the 7C-aglycone is not a product of dihydrophylloquinone catabolism, its continued fall over 25 d of phylloquinone depletion suggests that this period had been insufficient to establish a final steady state for this low level of phylloquinone intake, assuming that there had been no changes in menaquinone intake or intestinal synthesis. The absence of a steady state, together with evidence that the majority of urinary metabolites during phylloquinone restriction derives from nondietary sources, is in agreement with the conclusions of Olson and co-workers (14), that there is a body pool with a very slow turnover rate that may represent adipose tissue.

The dose-adjusted urinary excretion of the 5C-aglycone after dihydrophylloquinone repletion was significantly greater than that derived from phylloquinone, suggesting discrete bioavailability and/or turnover differences between these very similar vitamin K compounds. Plasma levels of dihydrophylloquinone were lower than that of phylloquinone for equivalent dietary intakes (10), suggestive of a lower intestinal absorption and/or a faster metabolic clearance rate. Data presented here of the greater relative excretion of dihydrophylloquinone are supportive of the latter. Whether the different clearance rates of these 2 compounds is a consequence of preferential conservation of phylloquinone for the replenishment of diminished vitamin K tissue stores is unclear. The inferior γ-carboxylation potency of dihydrophylloquinone, relative to phylloquinone, has previously been described in humans. In this study, whereas dihydrophylloquinone partially restored the impaired γ-carboxylation of hepatic prothrombin, it had no measurable effect on the γ-carboxylation status of bone osteocalcin (10). Another difference between phylloquinone and dihydrophylloquinone is that a proportion of dietary phylloquinone is converted to menaquinone-4 in vivo (21,22), whereas dihydrophylloquinone is not (23). Although the biological importance of this conversion is not understood, the tissue-specific storage of the resultant menaquinone-4 (24) may offer another explanation for the relatively higher retention of phylloquinone in the body, compared with dihydrophylloquinone.

We conclude that the assessment of urinary vitamin K excretion represents a new candidate marker of vitamin K dietary exposure and status. Importantly, it is noninvasive and the first biomarker that reflects the turnover and excretion of all K vitamins. At the present time, the only available indicator of vitamin K status that may be said to directly reflect storage and transport is serum phylloquinone. Other markers such, as PIVKA II, undercarboxylated osteocalcin, or the less-commonly measured, urinary free Gla may be said to be functional indicators. Serum phylloquinone has a number of drawbacks as a status biomarker, including its association with serum lipids, and the fact that it does not reflect the metabolic conversion of dietary phylloquinone to menaquinone-4 in humans. Most importantly, serum phylloquinone measurements reflect only one of several vitamin K compounds that contribute to vitamin K status. In contrast, the measurement of urinary vitamin K metabolites reflects excretion of all nutritionally relevant K vitamins, including any metabolic conversion of phylloquinone to menaquinone-4.

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Literature Cited

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