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HPLC and GC/MS determination of deuterated vitamin K (phylloquinone) in human serum after ingestion of deuterium-labeled broccoli☆

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Abstract

The ability to intrinsically label plant constituents with stable isotopes has the potential to advance the study of vitamin K-absorption and metabolism in humans. Broccoli, a primary food source of phylloquinone (VK-1), was grown hydroponically using 31 atom % deuterium oxide in order to label VK-1 within the food matrix. Deuterium-labeled broccoli (115 g; 168 μg VK-1) was fed to one male subject in a single serving. Multiple serum samples were drawn throughout the subsequent 24-hr period. Reversed-phase HPLC was used to extract and purify VK-1 in both broccoli and serum. Ion abundances of the deuterium-labeled and unlabeled (endogenous) VK-1 were determined using GC/MS in negative chemical ionization mode. No sample derivatization was required. Endogenous VK-1 produced isotopomers from m/z 450 to m/z 453. The labeled VK-1 isotopomers in the broccoli were from m/z 452 to m/z 467, with the most abundant isotopomer being m/z 458 (14.1% of total labeled VK-1). The GC/MS chromatograms from serum revealed both endogenous VK-1 and VK-1 derived from the deuterium-labeled broccoli. The profile of labeled VK-1 isotopomers in serum was identical to the VK-1 isotopomer profile in labeled broccoli, indicating that no deuterium was lost due to exchange either in the body or in sample preparation. At 4 hr following broccoli intake, there was an 81.1% enrichment of phylloquinone in serum; labeled VK-1 was no longer detectable in serum at 24 hr. Use of isotope labeled vegetables enables one to discriminate exogenous intake of VK-1 from endogenous pools and ultimately to determine bioavailability of VK-1 from foods.

Keywords: Vitamin K; Stable isotope; Phylloquinone; Absorption

1. Introduction

Vitamin K has an established role as a cofactor for the post-translational synthesis of γ-carboxyglutamic acid residues in at least 12 known vitamin K-dependent proteins [1]. In addition to its well-established role in blood coagulation, vitamin K has been identified as a potentially modifiable dietary factor in the prevention of osteoporosis [2]. Phylloquinone, also known as vitamin K_1 (VK-1), is the primary dietary source of vitamin K and as a corollary, the primary form in circulation (Fig. 1) [3]. Endogenous plasma or serum phylloquinone is in low concentrations relative to lipids and other lipid-soluble compounds (normal range: 0.3–2.6 nmol/L [4]). It has only been during the last two decades, through the use of high-performance liquid chromatography (HPLC) that the direct measurement of phylloquinone using low sample volumes of biological material has been possible [5]. Despite these analytical advances, the fundamentals of vitamin K metabolism, in particular the absorption and turnover of vitamin K obtained from different dietary sources are not well understood.

Several studies have defined absorption from phylloquinone-rich sources, such as green, leafy vegetables and certain plant oils, as the area under the plasma phylloquinone curve (AUC) [6,7]. Whereas AUC, as measured by HPLC,

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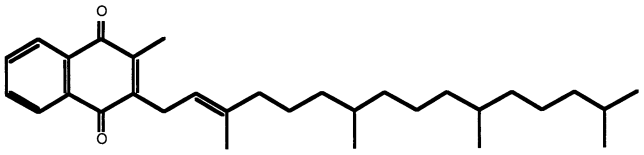

Fig. 1. Structure of Phylloquinone (MW 450).

is suitable for measuring absorption of phylloquinone from a single food, the rate of clearance from circulation is more difficult to measure, particularly as fasting phylloquinone concentrations are very low. Shearer et al [8] showed that tritiated phylloquinone was associated with the chylomicrons, when this compound was given orally to two healthy men. The clearance of phylloquinone from plasma was resolved as a fast initial pool, followed by a second slower pool. However, the potential hazard of ionizing radiation has limited the use of radioactive isotopes in human metabolic studies. Conversely, stable isotopes are nonradioactive forms of elements that naturally occur within the environment and have application for human studies. These isotopes can be separated and quantified by mass spectrometry (MS). There has been much recent work published using stable isotope labeled vitamins and pro-vitamins to study bioavailability and metabolic pathways *in vivo* using mass spectrometry [9–17]. To study phylloquinone absorption and clearance at physiological doses, we have developed a stable isotope-labeled technique using gas chromatography/mass spectrometry (GC/MS) and HPLC to measure the deuterated phylloquinone enrichment in human serum after ingestion of deuterium-labeled broccoli.

2. Materials and methods

2.1. Deuterium-labeled broccoli

Labeled and unlabeled broccoli plants (cultivar 'Emperor', F_1 hybrid) were grown hydroponically in an environmental growth chamber (Conviron Model PGW36; Winnipeg, Manitoba, Canada) at the USDA/ARS Children's Nutrition Research Center in Houston, Texas. All plants were maintained on a 16-h, 20°C/8-h, 15°C day/night regime and 70% relative humidity. Light was provided during the day from a combination of fluorescent and incandescent lamps; intensity of photosynthetically active radiation was 500 μ mol of photons m^{-2} s^{-1} at the top of mature plants. Plants were started from seeds that were germinated on filter paper for 6 days; seedlings were planted in polyethylene cups as previously described [18]. Plants were grown hydroponically (one plant per container) in 3.5 L of nutrient solution containing the following macronutrients in mM: KNO_3, 0.5; $Ca(NO_3)_2$, 0.5; KH_2PO_4, 0.25; $MgSO_4$, 0.25; and the following micronutrients in μM: $CaCl_2$, 25; H_3BO_3, 25: $MnSO_4$, 2; $ZnSO_4$, 0.2; $CuSO_4$, 0.5; H_2MoO_4, 0.5; $NiSO_4$, 0.1. Iron was added in chelated form as Fe(III)-EDDHA (N,N′-ethylenebis[2-(2-hydroxyphenyl)-glycine]) at 5 μM. MES buffer (adjusted with KOH) was added at 2 mM to maintain nutrient solution pH between 5.4 and 5.8. Solutions were changed weekly for unlabeled plants throughout the three months of growth, and for labeled plants during the first two months of growth. At 2 months of age, plants selected for deuterium enrichment were transferred to nutrient solutions with the mineral composition noted above, but in a medium containing 31 atom% heavy water (D_2O). For the 4 weeks of growth on heavy water, during which time head formation occurred in these plants, solutions were not replaced, but rather were topped off with additional solution (to 3.5 L when needed) as the plants consumed minerals and water. All nutrient solutions were constantly aerated with a bubbling air stream to provide oxygen to the roots; heavy water solutions were bubbled with an air supply containing no water vapor.

At approximately 3 months of age, broccoli heads of standard harvest size (comprised of immature florets and subtending stalk) [19] were cut from each plant. These were sealed in two layers of plastic zip-lock bags and were frozen and stored at –70°C. Once all heads were harvested, labeled and unlabeled broccoli were shipped on dry ice to the Vitamin K Laboratory at the Jean Mayer USDA-HNRCA at Tufts University and stored at –70°C until subsequent use.

Processing of labeled and unlabeled broccoli involved steaming for 5 min, immersion in cold deionized water (broccoli/water = 1:10) for 2 min, and draining. After the broccoli was well drained, heads were pureed to homogenize the samples; one aliquot (115 g) of processed labeled broccoli was used for subject consumption, and additional aliquots of labeled and unlabeled samples were used for determination of phylloquinone content by HPLC and GC/MS.

2.2. Metabolic study

A 23-year old male subject was recruited from the general population. The research protocol was approved by the Tufts University Human Investigation Review Committee, and written informed consent was obtained. The subject resided in the Metabolic Research Unit at the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University for two days. The residency period corresponded to a 24-hr absorption period following consumption of a single dose of deuterium-labeled phylloquinone in broccoli. At 8am on day 1 of the study, the subject was fed 115 g of steamed deuterium-labeled broccoli, in addition to a breakfast that consisted of 850 calories and 31% of energy from fat. Twelve mL blood samples were drawn at each time point of 0, 1, 2, 3, 4, 5, 5.5, 6, 6.5, 7, 7.5, 8, 10, 12, 16, 20, and 24 hr, with time 0 corresponding to the fasting blood draw at 8am, immediately prior to breakfast. The subject was fed a second and third meal following blood draws at 5.5 h and 8 h, respectively. Neither of these latter two meals

contained broccoli or other vitamin K-rich sources. Upon collection, the blood samples were centrifuged immediately at 1800g and 4°C for 20 min, and stored at –70°C for further analysis. Serum triacylglycerides were analyzed on a CO-BAS Mira (Roche Instruments, Belleville, NJ).

2.3. HPLC determination of phylloquinone concentrations

Phylloquinone used for standards was purchased (Sigma, St. Louis, MO) and the internal standard, $K_{1(25)}$ was a gift (Hoffman La Roche, Basel, Switzerland). Solvents used for extraction and chromatography were HPLC grade, and purchased from Fisher Scientific (Springfield, NJ). Working standards were prepared in HPLC grade methanol, and were characterized spectrophotometrically and chromatographically prior to use. All standards were stored at 2–8°C and protected from light and all operations were performed under yellow lighting. All glassware was rinsed with acetone prior to use to prevent contamination of samples with fluorescent material.

2.4. Broccoli sample preparation

The phylloquinone concentration of broccoli was measured using a modification of the procedure described by Booth and Sadowski [20]. Briefly, the procedure consisted of homogenizing the steamed broccoli in a commercial food processor (Waring Products Division, New Hartford, CT), removing about 1 g, and grinding it to a fine paste with a mortar and pestle. About 0.4 g broccoli paste was weighed into a 50 mL polypropylene centrifuge tube before adding 10 mL of deionized water, 15 mL propanol/hexane (3:2) and an appropriate amount of $K_{1(25)}$ internal standard. Cells were further disrupted by sonication for 1 min using a 1/8 inch tapered microtip with a Cell Disrupter Sonifier 350, (Branson Ultrasonics Corp., Danbury CT) set at output control 4 and 50% duty cycle. The sonicated sample was mixed for 10 min using a Multi-Tube Vortexer and centrifuged for 5 min at 1800xg to separate the phases. The hexane (top) layer was transferred to a glass culture tube and evaporated to dryness under reduced pressure in a centrifugal evaporator (Savant Instruments, Farmingdale, NY model Speed Vac SC210A). Ten [10] mL of hexane was then added to dissolve the residue.

The extracted phylloquinone was further purified using solid phase extraction (SPE). A 100 μL aliquot of the hexane solution was pipetted into a 12 X 75 mm test tube containing 0.5 mL of hexane. The SPE was performed using an ASPEC XL4 SPE instrument system (Gilson Medical Electronics, Inc., Middleton, WI). The ASPEC instrument was programmed to: 1) condition each disposable 3 mL (500 mg) Bond Elut silica column (Varian, Inc. Walnut Creek, CA) with 2.5 mL ethyl ether/hexane (3.5:96.5 v/v), followed by 2.5 mL of hexane, 2) load sample onto the column, 3) wash with 2.5 mL of hexane, and 4) elute sample into clean test tubes with 5 mL ethyl ether/hexane. The eluate was evaporated to dryness in the centrifugal evaporator. The residue was reconstituted initially in 30 μL of methylene chloride, followed by 170 μL of methanol containing 10 mM zinc chloride, 5 mM acetic acid, and 5 mM sodium acetate. A sample volume of 50 μL was injected into the HPLC.

2.5. Serum sample preparation

The concentration of serum phylloquinone was measured using a modification of the procedure described by Davidson and Sadowski [5]. Briefly, the procedure consisted of pipetting 0.25 mL of plasma into a 16 x 100 mm borosilicate culture tube with a screw cap. An appropriate amount of internal standard was added to each sample, followed by 0.5 mL of ethanol. The tubes were mixed for 5 s to denature proteins and 0.5 mL of deionized water and 1.5 mL of hexane were added. The tubes were capped and mixed for 2 min using a Multi-Tube Vortexer. The samples were centrifuged for 5 min at 1800xg to separate the phases. The hexane (top) layer was transferred to a glass culture tube and evaporated to dryness under reduced pressure in the centrifugal evaporator. Solid-phase extraction (SPE) was performed as previously described for phylloquinone determination of broccoli. A sample volume of 150 μL was injected into the HPLC.

2.6. HPLC procedure

The equipment used consisted of a Model 2690 Separations Module (Waters Corp, Milford, MA) equipped with a vacuum degasser using Millennium [32] software, version 3.05.01 for peak integration and sample concentration calculations. The analytical column (150 x 3 mm i.d.) was packed with 5 micron BDS Hypersil C18 (Keystone Scientific, Bellefonte, PA). The post column reactor consisted of a stainless steel column (50 x 2.0 mm i.d.) dry packed with zinc (-200 mesh) purchased from Johnson Matthey Electronics (Ward Hill, MA). Fluorescence was monitored with a model RF-10AXL Shimadzu Fluorescence Detector using a 12 μL quartz flow cell (Shimadzu Instruments, Columbia, MD). The mobile phase was composed of methanol, to which 5.5 mL of aqueous solution (2 M zinc chloride, 1 M acetic acid, and 1 M sodium acetate) per L was added (solvent A), and methylene chloride (solvent B) (90:10 v/v). A gradient elution procedure was programmed as follows: a solution of 90% solvent A and 10% solvent B was pumped at 0.60 mL/min for the first 11.5 min. For the next 8 min, the flow rate was increased to 0.80 mL/min, and the mobile phase composition was changed to 70:30 (A:B) to remove the more lipophilic compounds from the column. The mobile phase composition was then changed to 90:10 (A:B), and after 4.0 min, the flow rate was changed back to 0.60 mL/min to re-equilibrate the column. The cycle was finished at 24.0 min.

The excitation wavelength was 244 nm and emission was

monitored at 430 nm. A calibration standard was injected after every six samples in a run to compensate for potential drift in chromatographic conditions. Standard curves were prepared from each calibrator injection. The fluorescence responses for vitamin K_1 and for $K_{1(25)}$ are linear with the slope of the lines bisecting zero. Therefore, single-point calibration was routinely performed, forcing the slope of the line through zero. Quantitation was achieved by direct comparison of peak area ratios (K_1 to $K_{1(25)}$) generated from the calibration standard to those generated by the sample. The limit of detection for this assay was 10 pg per injection.

2.7. Characterization of phylloquinone using GC/MS

For both broccoli and serum samples dedicated for GC/MS analysis, the sample preparation procedures were the same as those described for HPLC analysis, with the following exceptions: 0.5 mL of serum was used; no internal standard was added to the initial samples; the post column reactor was removed from the HPLC; and the sample fraction eluting from 5.20–6.20 min was collected into a 16 x 100 mm borosilicate culture tube with a screw cap. Once collected, the phylloquinone was subsequently extracted from the eluate by adding 0.5 mL deionized water and 2 mL hexane into the tube and vortexed for 2 min. The mixture was centrifuged for 5 min at 1800xg to separate the phases and the hexane (top) layer was aspirated into a 12 x 75 mm test tube. The hexane was evaporated to dryness in a centrifugal evaporator. The residue was redissolved with 50 μL of hexane; vortexed for 5 s, and transferred into a brown glass sample vial. The GC/MS analysis was completed within 24 hr to prevent losses due to sample degradation.

2.8. GC/MS procedure

Phylloquinone isotopic ion abundance was determined using a Hewlett Packard 5890/5988A GC/MS, with a 7673A autoinjector. 1–2 μL of hexane containing 10–120 pg of phylloquinone were injected using a cool on-column injection port onto a uncoated 0.5m x 0.530mm fused silica column which was connected by a zero-dead volume connector (HP part no. 5061–5801) to a 30m x .25mm fused silica GC column coated with HP-5MS (Hewlett Packard). The GC oven temperature was programmed from 50–325°C at 25°C/min. and the flow rate of He through the GC column was held constant at 2.5 mL/min. Methane negative chemical ionization (0.5 torr of methane) was used to ionize the phylloquinone, and the temperature of ion source was 200°C. Methane negative ion chemical ionization produced only the negative molecular ions of phylloquinone (e.g. m/z 450 for the predominant endogenous molecule). Selected ion monitoring in the range from m/z 450–468 was employed to detect the isotopomers of phylloquinone in broccoli and serum. The data were analyzed with an HP Chemstation data system and further processed using a Microsoft Excel spreadsheet. By GC/MS, the detection limit of serum phylloquinone was 5 pg.

2.9. Deuterium enrichment of serum phylloquinone

The abundance of phylloquinone was determined by integrating the peak area under the mass chromatograms of the negative ions at m/z 450 to m/z 467. Calculation of the percentage enrichment of serum phylloquinone was based on the ion abundances of labeled and endogenous phylloquinone in serum using the following formula:

$$\% \text{ enrichment} = (\Sigma \text{ Abundance of labeled VK-1}/\Sigma \text{ Abundance of total VK-1}) \times 100$$

$$\% \text{ enrichment} = [(\text{m/z } 453 \text{ to } 467)/(\text{m/z } 450 \text{ to } 467)] \times 100$$

It was difficult to obtain accurate ion abundances for all the isotopomers of serum phylloquinone at every time point due to the dynamic range limitation of the GC/MS detection system. Instead, an average ratio of the abundance of the most predominant isotopomers to the abundance of all isotopomers was calculated using serum samples collected between 3 and 5 hr after consumption of the deuterium-labeled broccoli. These four time points (3h, 4h, 4.5h, and 5h) corresponded to the highest percentage of enrichment of serum phylloquinone. For endogenous serum phylloquinone, isotopomers at m/z 450 and 451 were selected as the most abundant isotopomers [ratio = (m/z 450 to 451)/(m/z 450 to 453)]; for deuterium-labeled serum phylloquinone, isotopomers m/z 456 to 460 were selected as the most abundant isotopomers to calculate the ratio [ratio = (m/z 456 to 460)/(m/z 453 to 467]. These ratios were then used to calculate the percentage enrichment at every time point for which the most abundant isotopomers were within detectable range.

The absolute concentrations of deuterium-labeled phylloquinone in serum were determined by multiplying the percentage deuterium enrichment of serum phylloquinone by the concentration of total phylloquinone as determined by HPLC analysis.

2.10. Statistics

A Pearson correlation between serum phylloquinone and triacylglyceride concentrations was performed using Systat (version 9, SPSS Inc., Chicago, IL).

3. Results and discussion

Broccoli heads, harvested from plants grown hydroponically in a medium enriched with 31% heavy water (D_2O) contained 146 ± 3.6 μg phylloquinone per 100g fresh

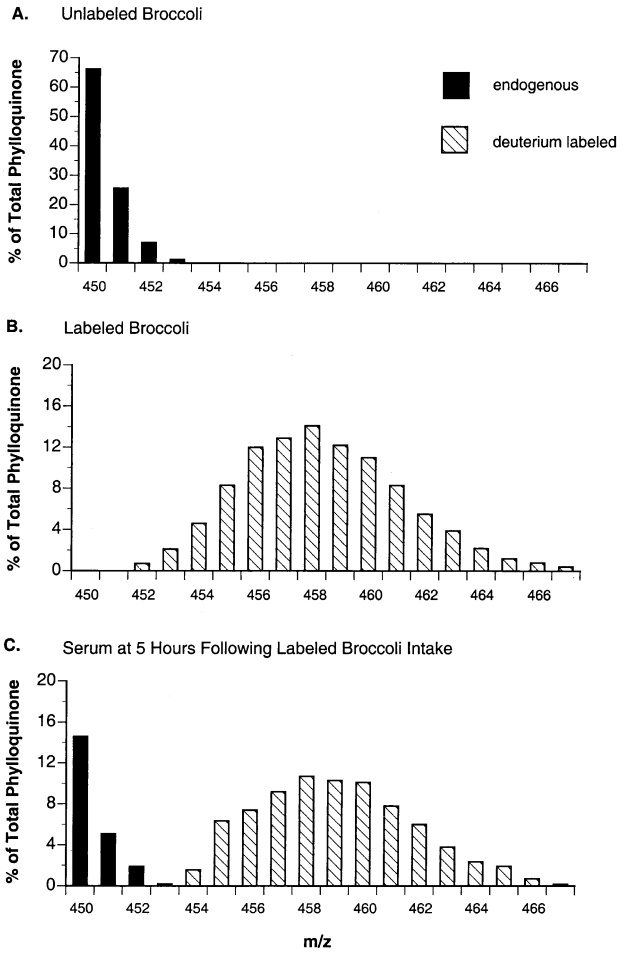

Fig. 2. Isotopomer Profiles in Unlabeled Broccoli (Fig. 2a), Labeled Broccoli (Fig. 2b), and in Serum at 5 hr Following Intake of Deuterium-Labeled Broccoli (Fig. 2c).

weight of steamed and pureed head (immature florets plus stalk). This value is within the range of published data for unlabeled broccoli [21]. When purified by HPLC and subsequently analyzed by GC/MS, the isotopomer profile of the deuterium-labeled phylloquinone in broccoli was from m/z 452 to 467 (Fig. 2b), indicating that deuterium atoms were located at 2 to 17 of the 46 hydrogen positions in individual phylloquinone molecules. The most abundant isotopomer was at m/z 458, which corresponded to 14.1% of the total labeled phylloquinone. Endogenous phylloquinone in broccoli had 4 isotopomers (m/z 450–453) (Fig. 2a); these result from the natural abundance levels of ^{13}C (1.1%) and deuterium (0.015%) that exist in nature, and which can be incorporated into phylloquinone molecules.

The deuterium-labeled broccoli that contained 168 μg of phylloquinone was fed to a single subject, and serum was drawn at varying intervals over the course of 24 hr. In serum samples collected 2–24 hr after consumption of deuterium-labeled broccoli (Fig. 2c), there were isotopomers that corresponded to both endogenous and labeled phylloquinone. This heterogeneous isotopomer profile in serum reflects both newly absorbed and previously existing phylloquinone in circulation. The isotopomer profile of labeled phylloquinone in serum (Fig. 2c) was consistent with the profiles of phylloquinone in the labeled broccoli (Fig. 2b). These results indicate that no deuterium was lost to exchange, either in the body or in sample preparation.

The percentage deuterium enrichment of serum phylloquinone is presented in Fig. 3. Deuterium-labeled phylloquinone was detected in serum within 2 hr of consuming the labeled broccoli, and reached a peak enrichment of 81% at 5 hr following consumption. An enrichment of ~80% was maintained between 4 and 6 hr, after which there was a rapid decline until 8 hr, followed by a slower decline. These data are consistent with previous studies, which report a

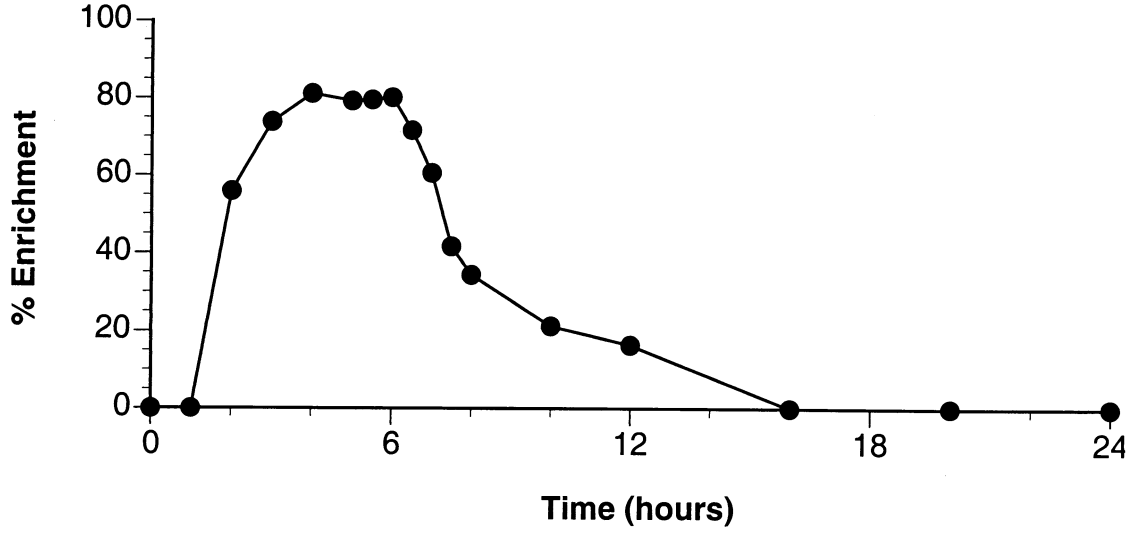

Fig. 3. Percent Deuterium Enrichment of Serum Phylloquinone.

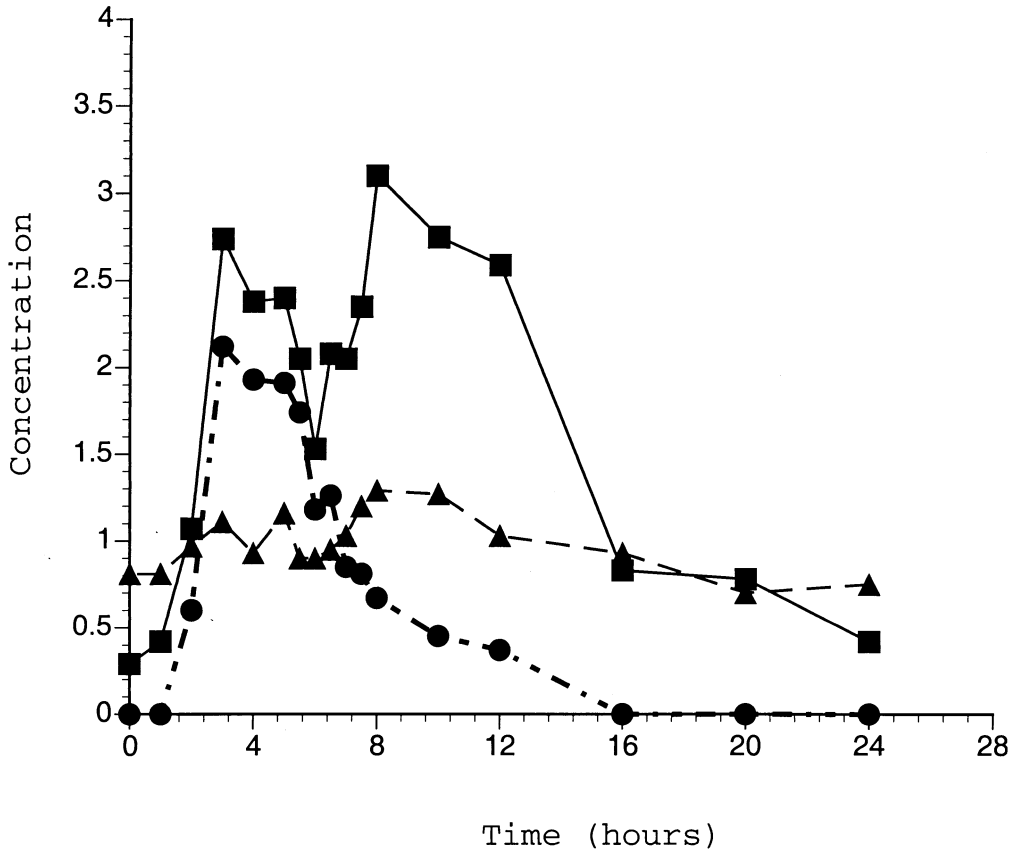

Fig. 4. Deuterium-labeled and endogenous serum phylloquinone concentrations following intake of deuterium-labeled broccoli. Deuterium-labeled phylloquinone (nmol/L) -●-; endogenous phylloquinone (nmol/L) -■-; triacylglycerol (mmol/L) -▲-.

second, slower clearance pool of phylloquinone using a radioactive tracer [8,22]. Some isotopomers between m/z 453 to m/z 467 were still detectable in serum more than 12 hr following consumption of the deuterium-labeled broccoli. However, other predominant isotopomers were no longer detectable so percentage enrichment could not be accurately calculated after 12 hr.

The serum total phylloquinone concentrations ranged from 0.29 to 3.10 nmol/L (Fig. 4), with an initial peak 3 hr following consumption of the deuterium-labeled broccoli. Although the dietary phylloquinone intakes from the second and third meals were low (total intake for breakfast, lunch and dinner meals, excluding the deuterium-labeled broccoli: 58 μg of phylloquinone), total serum phylloquinone concentrations peaked again at 8 hr following deuterated broccoli intake, presumably in response to postprandial increases in triacylglyeride concentrations. Total phylloquinone concentrations were highly correlated with corresponding triacylglyceride concentrations ($r = 0.84$), consistent with earlier reports [3,23]. In contrast, the deuterium-labeled phylloquinone in serum did not increase in response to fluctuations in triacylglycerides associated with intake of subsequent meals that did not contain labeled broccoli.

A caveat to this methodology study was the selection of a single young subject who had fasting serum phylloquinone concentrations (0 and 24 hr) that were at the lowest limit of normal range for this assay [4]. Although intakes of 377 μg of phylloquinone from broccoli can significantly increase total plasma phylloquinone concentrations within 24 hr [24], we did not observe a similar increase at 24 hr following intake of 168 μg of phylloquinone from broccoli plus 58 μg from the meals in the current study. By 12 hr following intake of the broccoli, the concentrations of the deuterium-labeled phylloquinone were approaching the limit of detection of the GC/MS, despite a 14.3% deuterium enrichment of serum phylloquinone. Young adults consistently have low circulating concentrations of phylloquinone [4,25], which have been attributed to lower lipid concentrations compared to older adults [4]. Therefore it is plausible that the turnover time of phylloquinone is at least 36 hr, as reported among studies in adults of a wider age range with average plasma or serum phylloquinone concentrations higher than reported in the current study [22,26]. The stable isotope technique presented here will provide a unique opportunity to examine potential age-related differences in vitamin K absorption and clearance in future studies using larger sample sizes.

In conclusion, we have developed an HPLC and GC/MS technique based on deuterium-labeled vitamin K for the

study of vitamin K absorption and clearance. We observed rapid absorption of vitamin K following intake of deuterium-labeled broccoli, consistent with other studies on vitamin K absorption [6,7,26,27]. Approximately 80% of the serum phylloquinone was enriched with deuterium at 5 hr following broccoli intake, after which there was a rapid decline which is suggestive of rapid uptake and clearance. Because the deuterated phylloquinone from broccoli can be measured separately from endogenous forms in serum and foods, this technique will provide the foundation for future studies on relative bioavailability of vitamin K from supplements and different dietary sources.

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