

Vitamin K absorption and kinetics in human subjects after consumption of ¹³C-labelled phylloquinone from kale

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(Received 8 October 2009 – Revised 10 January 2010 – Accepted 11 March 2010 – First published online 27 April 2010)

The absorption and plasma disappearance of vitamin K were investigated by uniformly labelling phylloquinone in kale with carbon-13, and by feeding the kale to study subjects. Seven healthy volunteers ingested a single 400 g serving of kale with 30 g vegetable oil. The kale provided 156 nmol of phylloquinone. Serial plasma samples were collected and analysed for the appearance of ¹³C-phylloquinone by HPLC–MS. Six of the subjects showed significant amounts of labelled phylloquinone in plasma, though one subject's plasma was not consistently enriched above the detection limit, and this subject's baseline plasma phylloquinone level was the lowest in the group. After ingestion of the labelled kale, plasma ¹³C-phylloquinone concentration increased rapidly to a peak between 6 and 10 h, and then rapidly decreased. Average peak plasma concentration for the six subjects with detectable ¹³C-phylloquinone was 2.1 nmol/l. Plasma concentration–time data were analysed by compartmental modelling. Modelling results demonstrated a mean (*n* 6) bioavailability of phylloquinone from kale to be 4.7%. Plasma and tissue half-times for phylloquinone were found to be 8.8 and 215 h, respectively.

Bioavailability: Stable isotopes: Mathematical modelling

Vitamin K refers to a group of compounds which can act as cofactors for the enzyme γ -glutamyl carboxylase. Its function is to facilitate post-translational conversion of specific glutamyl residues to γ -carboxyglutamyl residues in a number of proteins, particularly in several involved in the blood coagulation cascade. This enzyme also catalyses the carboxylation of osteocalcin in bone.

Phylloquinone, the major dietary form of vitamin K, is found predominantly in green, leafy vegetables. The bioavailability of purified phylloquinone has been reported to range from as low as 2% to as high as 80%^(1,2). Other studies have reported the relative bioavailability of food-bound phylloquinone compared to that of a supplement, with the conclusion that the bioavailability of phylloquinone from vegetables is significantly less than that from purified phylloquinone^(3–5), though no specific values for bioavailability of phylloquinone from vegetables have been reported. To determine the bioavailability of phylloquinone from a green, leafy vegetable, we conducted a study in which volunteers consumed isotopically labelled kale, and provided multiple blood samples for several weeks following the kale ingestion. A compartmental model was constructed to calculate the bioavailability of phylloquinone from kale, and to determine body pool size and rates of elimination.

Experimental methods

Isotopic labelling of kale

The method by which the nutrients in kale (*Brassica oleracea* var. *Acephala* cv. *Vates*) were labelled with ¹³C has been described previously⁽⁶⁾. Briefly, kale tissue was completely labelled with ¹³C by growing plants in a controlled environmental chamber with the only source of carbon being ¹³CO₂. A sealed clear acrylic box (Plexiglas G; Rohm and Haas, Wilmington, DE, USA) with a separate base was placed inside the controlled environmental chamber. The base and top of the acrylic box were fitted together via a water trough to provide a leak-proof seal, thus preventing exchange of ¹³CO₂ with atmospheric CO₂. Plants which were transferred into the growth chamber as seedlings were grown in Nalgene bins placed inside the acrylic box. Nutrient solution was delivered through tubes attached to the base portion of the acrylic box. Growth conditions were maintained as follows: temperature of 24°C; humidity of 90%, light with a 24 h photoperiod, 900 μ mol/m² per s of photosynthetically active radiation 400–700 nm, and the partial pressure of CO₂ (¹³CO₂, >99% carbon-13) inside the chamber was maintained at 36 Pa.

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Kale plants were harvested at leaf canopy closure (approximately 10 d). Roots and primary leaves were removed, and material for the feeding study was cleaned, weighed and blanched. The blanched kale was chopped into 1" × 1" pieces, mixed for batch uniformity and frozen at −80°C until prepared for consumption.

Subjects, study design, diet and sample collection

Seven healthy, non-smoking volunteers (four males and three females) from the Beltsville, MD, area participated in the present study. The present study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Johns Hopkins University Bloomberg School of Public Health Committee on Human Research. Written informed consent was obtained from all subjects. Subjects' characteristics (means and standard deviations) were 46 (SD 14) years of age, 71 (SD 8) kg body weight and BMI of 25 (SD 3) kg/m².

Each subject consumed a single 400 g (three cups) serving of ¹³C-labelled kale. Before consumption, frozen kale was transferred to a refrigerator (6°C) to thaw for 12 h, and then warmed in a 900 W microwave oven for 2 min. We administered the kale to a single individual as a pilot test to ensure that the nutrients were elevated above the detection limit before proceeding with the remaining subjects. The kale was consumed with 30 g peanut oil (*n* 1, pilot subject) or 30 g safflower oil (*n* 6). The kale treatment provided 156 nmol phyloquinone (equivalent to 70 µg unlabelled phyloquinone). The peanut oil contained 0.2 µg phyloquinone per 30 g and the safflower oil contained 2.1 µg phyloquinone per 30 g, with both oils contributing minimal vitamin K compared with the kale treatment⁽⁷⁾.

Subjects were provided a controlled diet for 1 week before kale ingestion and throughout the collection period. All foods were provided by the Beltsville Human Nutrition Research Center, and subjects were instructed to consume all foods and only foods provided by the Nutrition Center. The diet contained 15 % of energy from protein, 32 % from fat and the remainder from carbohydrate. The vitamin K content of the diet depended on the energy level of the subjects, and provided 119 µg vitamin K per 8.4 MJ (2000 kcal)⁽⁷⁾. Vitamins and supplements were prohibited throughout the study. On the ¹³C-kale treatment day, lunch and dinner were provided at 5 and 10 h after the treatment.

Blood collection times were as follows: ten collections across the treatment day, two collections (AM and PM) on the following day, each morning for the next 3 d (fasting) and semi-weekly for the next 3 weeks (fasting). Blood samples were collected into vacutainers containing EDTA, and were centrifuged at 2560 *g* for 10 min. Plasma aliquots of 1 ml were stored in cryovials at −80°C until analysis.

Reagents

Chloroform, ethanol, hexane, isopropanol, methanol and methyl tertiary butyl ether were purchased from Fisher Scientific (Pittsburgh, PA, USA). Phyloquinone, β-apo-8'-carotenol and butylated hydroxytoluene were purchased from Sigma Chemical Company (St Louis, MO, USA). ¹³CO₂ (>99 % carbon-13) was purchased from Isotec, Inc. (Miami, OH, USA).

Kale extraction

Kale was ground into a fine powder with liquid N₂ using a mortar and pestle. Three-gram samples of powder were weighed into each of two 25 ml test-tubes for extraction, and 0.0178 µg of β-apo-8'-carotenol was added as an internal standard. Protein was precipitated with 9 ml of 0.1 % butylated hydroxytoluene in ethanol and vortex-mixed for 2 min. Hexane–toluene (5:4, v/v, 9 ml) was added and the samples were vortex-mixed and then placed in a 70°C water-bath for 10 min. After the addition of 180 µl of 80 % KOH, the samples were vortex-mixed and returned to the water-bath for 15 min, with vortex-mixing for 8 min. The samples were then placed on ice, and 3 ml of double-distilled water were added before vortex-mixing again. After sample centrifugation at 1140 *g* for 10 min, the organic layer was transferred to a separate test-tube. The extraction was repeated two additional times with 6 ml hexane–toluene (10:8, v/v). A 100 µl aliquot was taken from the combined organic layer, dried under N₂ and reconstituted in 200 µl of methyl tertiary butyl ether–methanol (1:1, v/v) before injection onto the liquid chromatography–MS apparatus.

Plasma extraction

The plasma analysis procedure used was the one optimised for simultaneous measurement of carotenoids, retinol and phyloquinone⁽⁶⁾. Phyloquinone and ¹³C₃₁-phyloquinone were extracted from 0.5 ml of plasma. Plasma was thawed, and β-apo-8'-carotenol (50 µl of 0.356 µg/ml) was added as an internal standard immediately before the addition of 0.5 ml of ethanol for precipitation of protein. Samples were extracted twice with 1.5 ml hexane. The combined hexane extracts were dried under N₂, and reconstituted in 200 µl of methyl tertiary butyl ether–methanol (1:1, v/v) before injection onto the liquid chromatography–MS apparatus.

Liquid chromatography–MS conditions

An Agilent Technologies (Palo Alto, CA, USA) 1100 series liquid chromatography instrument was used. The instrument included a cooled autosampler, automatic solvent degasser, binary pump, cooled column compartment, a G1315A diode array detector and a G1946A mass spec detector with an atmospheric pressure chemical ionisation source. An YMC C₃₀ column (3 µm, 250 × 4.6 mm) and C₃₀ guard cartridge were used. The mass spec detector source conditions were as follows: spray chamber gas temperature set at 350°C, vaporiser temperature set at 400°C, nitrogen nebuliser pressure set at 45 psig (310 kPa) and corona current set at 5 µA. Initially, the VCap was set to 3800 V and the flow rate of the drying gas (nitrogen) was set to 7 l/min; at 7.5 min, the VCap changed to 3600 V and flow rate of the drying gas to 6 l/min. Selected ion monitoring was used for the detection of protonated unlabelled phyloquinone and ¹³C₃₁-phyloquinone (*m/z* 451 and 482) and internal standard β-apo-8'-carotenol (*m/z* 417). Data were collected using the Agilent Chemstation software. Molar concentrations of labelled and unlabelled phyloquinone were calculated based on a response curve for β-apo-8'-carotenol v. unlabelled phyloquinone. The limit of detection and limit of quantification for phyloquinone were 3 and 10 fmol, respectively.

The solvent system consisted of 1 mM-ammonium acetate in methanol (solvent A) and methyl tertiary butyl ether (solvent B). A solvent gradient was used in which the mobile phase consisted of 15% solvent B at 0 min, which increased linearly to 30% solvent B at 12 min. The mobile phase was held at 30% solvent B until 18 min. Then, solvent B was increased to 100% and held until 23 min, and was then decreased back to 15% for 10 min for column re-equilibration. The injection volume was 50 μ l, and the flow rate was 1 ml/min.

Calculations

Compartmental modelling was performed using the WinSAAM software package. A three-compartment catenary model was constructed with compartments for the gastrointestinal tract (compartment 1), the plasma (compartment 2) and a body tissue pool (compartment 3), and initial transfer parameters were estimated based on the features of the plasma phylloquinone concentration–time curve. Parameters were then adjusted in physiologically compatible ways to improve the fit of the model prediction to observed data values. Once the model and parameters provided good visual fits to the ^{13}C -phyloquinone data, a least-squares procedure was used to minimise the difference between model prediction and observed data. Each subject was modelled individually. The Extended Multiple Studies Analysis feature in WinSAAM was used to analyse the group of subjects as a whole.

Results

Six of the subjects showed significant amounts of labelled phylloquinone in plasma, though one subject's plasma was not consistently enriched above the detection limit, and this subject's baseline plasma phylloquinone level was the lowest in the group. Of the subjects who responded to the kale, labelled phylloquinone was first detected in the plasma at 3 h after kale ingestion (Fig. 1) and it was observed throughout the 28 d sampling period for three subjects, and for shorter

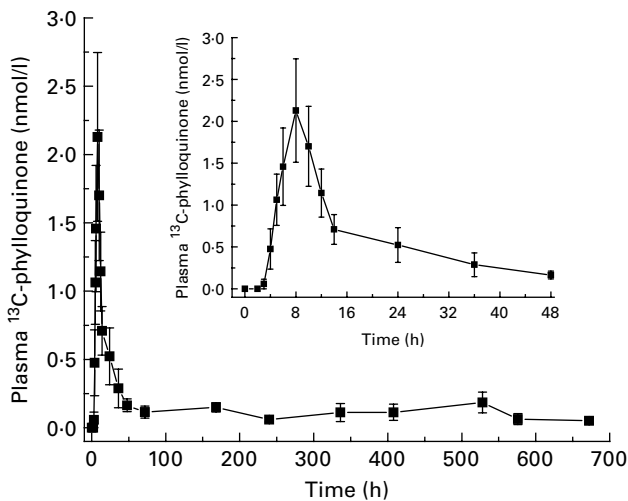


Fig. 1. Mean plasma ^{13}C -phyloquinone concentration (nmol/l) as a function of time after ingestion of labelled kale. Inset shows the same data for the first 72 h of sampling. Data represent mean values with their standard errors for the six subjects whose plasma values rose above the quantification limit.

periods for the other three subjects (through day 24 for one subject, through day 22 for one subject and through day 7 for one subject) (Fig. 1). The time of peak plasma concentration ranged between 6 and 10 h. Mean peak ^{13}C -phyloquinone concentration in plasma was 2.12 nmol/l (range 0.37–5.1 nmol/l, n 6). In all cases, there was a sharp rise in plasma ^{13}C -phyloquinone following kale ingestion, followed by a sharp decline, and then maintenance of plasma ^{13}C -phyloquinone at a low level thereafter.

A three-compartment catenary model was used to analyse the pharmacokinetics of the plasma ^{13}C -phyloquinone for the six subjects whose plasma levels rose significantly. This model provided a good prediction of plasma phylloquinone levels for each subject, with good statistical certainty on fractional transfer coefficients between compartments (all CV values for transfer coefficients were less than 20%). The model prediction compared with experimentally measured plasma ^{13}C -phyloquinone values for a representative subject is shown in Fig. 2. Pharmacokinetic analysis provided values for bioavailability, mass of vitamin K absorbed from kale and half-time values (Table 1). The bioavailability of phylloquinone from kale was found to be on average 4.7%, and it ranged from 1 to 14%. Thus, on average, 7.2 nmol (3.2 μ g) of the 156 nmol phylloquinone ingested in the labelled kale were absorbed into the systemic circulation.

Using the model, the vitamin K utilisation rate and tissue storage pool were calculated for a man weighing 86 kg, which is the mean body weight for an adult male in the United States⁽⁸⁾, and a woman weighing 74 kg, which is the mean body weight for an adult female in the United States⁽⁸⁾, with plasma phylloquinone levels of 1.43 and 1.47 nmol, respectively⁽⁹⁾. The tissue storage pools for the representative man and woman were found to be 103 nmol (46 μ g) and 91 nmol (41 μ g), respectively.

Discussion

A number of studies previously have used purified, isotopically labelled phylloquinone to investigate vitamin K kinetics in human subjects. Phylloquinone has been labelled with

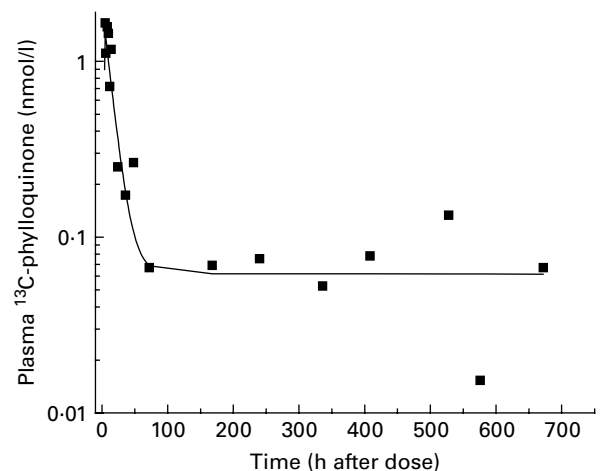


Fig. 2. Model-predicted (—) and observed (■) plasma ^{13}C -phyloquinone concentration for a representative subject. The model prediction is in good agreement with observed data.

Table 1. Results from pharmacokinetic modelling*
(Mean values and standard deviations)

	Mean	SD
Mean bioavailability (%)	4.7	4.8
Mean mass of vitamin K absorbed from 400 g kale (nmol)	7.2	7.5
Plasma half-time (h)	8.8	3.8
Plasma decay coefficient (%/h)	9.1	3.8
Tissue half-time (h)	215	132
Tissue decay coefficient (%/h)	0.014	0.017

* Plasma ^{13}C -phyloquinone concentrations of six subjects rose above the quantification limit.

^3H ^(2,10–14), carbon-13^(1,15) and ^2H ⁽¹⁾. Oral doses have ranged from 3 μg (3 times/d for 3 d) to 1 mg (as a single bolus dose)^(1,2,12,15). Other studies have involved intravenous injections of labelled vitamin K^(1,13,14). Sampling times after dosing have ranged from 7 h to 6 d^(1,2,10–12,14,15). In addition, two studies have involved labelled phyloquinone delivered in a plant matrix. Dolnikowski *et al.*⁽¹⁶⁾ showed feasibility by administering broccoli labelled with ^2H to a single male subject and collecting blood for 24 h. Erkkila *et al.*⁽¹⁷⁾ labelled collard greens with ^2H , fed the product to volunteers and measured labelled phyloquinone in fractionated plasma for 72 h after ingestion of the dose.

The previous studies with labelled phyloquinone, as well as some with unlabelled phyloquinone, have provided limited information about phyloquinone bioavailability. These studies have generally involved evaluation of the area under the plasma concentration–time curve after the volunteers ingested vitamin K in the form of vegetables, supplements or fortified oil^(3–5,18). Overall, these studies have provided bioavailability estimates of purified vitamin K ranging widely from 2 to 80%. The dual-isotope method for phyloquinone bioavailability published by Jones *et al.*⁽¹⁾ suggested that the bioavailability of purified phyloquinone was 13 (SD 9)%. Previous studies have also suggested that the food matrix inhibits absorption of vitamin K, as plasma response after ingestion of vitamin K from plant-based foods has been lower than that for purified vitamin K^(3–5,19). Jones *et al.*⁽¹⁵⁾ demonstrated that different meal components ingested with phyloquinone can significantly impact bioavailability. Gijsbers *et al.*⁽⁴⁾ compared the bioavailability of phyloquinone from spinach to that from a pharmaceutical preparation. By comparing the plasma phyloquinone response after each treatment, they concluded that relative bioavailability of spinach was 4–12% of that of the supplement. Since the reports of phyloquinone bioavailability have been widely ranging, this could correspond to a bioavailability from spinach of 0.1–10%. The present study suggests a mean bioavailability of phyloquinone from kale of 4.7%, with a range of 1–14%.

The inter-individual variability in phyloquinone bioavailability reported here is slightly higher than that found in a previous study of purified phyloquinone⁽¹⁾. Jones *et al.*⁽¹⁾ reported a larger range of bioavailability (2–26%) than that we observed (1–14%), though the CV for the earlier study was 69%, while the CV found in the present study was 100%. The study of Jones *et al.*⁽¹⁾ involved a larger number of subjects, which would be expected to reduce the CV compared to that of the present study involving fewer

volunteers. In addition, differences in the ability to digest the plant matrix would be expected to increase the variability in the present study compared to a study of purified phyloquinone. Still, the inter-individual variability of the two studies is similar.

Several previous studies have estimated vitamin K tissue pool size by extrapolating the terminal decay curve back to its intercept. Jones *et al.*⁽¹⁾ predicted a total body pool size of 5.1 nmol (2.3 μg), while Olson *et al.*⁽¹³⁾ predicted an average pool size of 194 nmol (87.6 μg) for moderate vitamin K intake levels and 99 nmol (44.7 μg) after several weeks of low vitamin K intake. We used compartmental modelling to simulate pool sizes, given the plasma phyloquinone concentrations found in the older population⁽⁹⁾ and the mean body weights for men and women⁽⁸⁾ in the US. Our calculations suggest that an average man and woman would have 103 nmol (46 μg) and 91 nmol (41 μg) of stored phyloquinone, respectively. These are in accordance with the values estimated by Olson *et al.*⁽¹³⁾.

Several of the previous studies have reported half-times for vitamin K disappearance, suggesting two phases of decay: one with a faster half-time, ranging from 0.21 to 1.0 h, and the other with a slower half-time, ranging from 1.8 to 3.4 h^(1,11,12,14). These studies were fairly short in duration, ranging from 6 to 10 h; thus, only early kinetic behaviour could be monitored. Olson *et al.*⁽¹³⁾ conducted a kinetic study of longer duration (6 d) to find substantially longer half-times of 1.0 and 27.6 h, with the latter half-time being similar to the 22.8 h reported by Erkkila *et al.*⁽¹⁷⁾ for a study of labelled collard greens. The present study extended measurements for several weeks after kale ingestion, allowing us to detect a significantly slower elimination curve than the curve that had been observed previously and producing a half-time for tissue elimination of 215 h. While this is substantially longer than the values reported for the shorter duration studies of plasma kinetics, it is substantially shorter than what would be predicted from analysis of tissue pools predicted in Olson's study⁽¹³⁾. Olson *et al.*⁽¹³⁾ conducted the kinetic study under conditions of high and low phyloquinone intake. From the plasma decay curves, pool sizes were calculated by extrapolating the curve back to its intercept for moderate vitamin K intake and after several weeks of low vitamin K intake. Olson *et al.*⁽¹³⁾ derived an equation for vitamin K pool size based on phyloquinone plasma levels. Before depletion, Olson reported a plasma phyloquinone concentration of 1.94 nmol/l on average, which would correspond to a body pool of 99 μg for a man weighing 86 kg, the average body weight of an adult male in the United States⁽⁸⁾. After depletion, plasma concentration dropped to 1.05 nmol/l, corresponding to a pool size of 21.5 μg . The pool was depleted 78% over the 66 d. In general, 75% depletion corresponds to two half-times. Thus, if two half-times occurred over approximately 66 d, then one half-time is 33 d or 792 h.

The model presented here describes the kinetics of vitamin K in the form of phyloquinone. The primary circulating form of vitamin K in blood is phyloquinone. Shearer *et al.*⁽²⁾ found that 80% of radioactivity in plasma after consumption of an oral dose of tritiated phyloquinone was in the form of phyloquinone at the peak. Olson *et al.*⁽¹³⁾ reported that 2 h after administering tritiated phyloquinone to volunteers, 90% of the radioactivity in the plasma was associated with authentic

phylloquinone. In the model, conversion of phylloquinone to metabolites would be captured in the calculation of elimination of phylloquinone from plasma. However, the kinetics of specific metabolites is not included in this analysis.

In conclusion, the administration of ^{13}C -phylloquinone to adult volunteers and subsequent compartmental analysis have revealed new information about bioavailability, pool size and elimination of vitamin K. Bioavailability of vitamin K from kale is low, pool sizes may be larger than those previously estimated and measurement of terminal elimination rates requires sampling times over several weeks.

Acknowledgements

The authors' contributions are as follows: J. A. N. and B. A. C. designed the study; S. J. B. produced the labelled plant material; A. C. K. developed and conducted laboratory analysis; J. A. N. and D. J. B. interpreted data; and J. A. N. wrote the manuscript. The present work was funded by the U.S. Department of Agriculture. There are no conflicts of interest related to the present work. The present research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

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